IMPACT OF ACUTE SCD1 INHIBITION ON PLASMA LIPIDS AND ITS EFFECT ON NUTRIENT HANDLING AND INSULIN SIGNALING IN MURINE SKELETAL MUSCLE

Jaclyn M. Omar

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

Department of Human Nutritional Sciences

University of Manitoba

Winnipeg, Manitoba, Canada

ABSTRACT

Stearoyl-coA desaturase-1 (SCD1) activity has been linked to the development of obesity and the metabolic syndrome (MetS) through its central role in lipid metabolism. Understanding how changes in SCD1 activity affect obesity and MetS risk biomarkers and investigating how these changes in activity affect nutrient handling in non-hepatic tissues is also important.

This study investigated how acute SCD1 inhibition effected plasma lipids, skeletal muscle nutrient handling and insulin signaling in mice fed a high-carbohydrate very-low fat diet for 10 weeks. This study demonstrated that SCD1 inhibition created acute dyslipidemia, altered nutrient handling protein activity and increased the percentage of saturated fatty acids (SFA) in hepatic and muscle tissue, independent of dietary oleic acid content. However, the molecular controls of protein synthesis in the mTOR pathway were not affected by the loss of SCD1 activity.

In conclusion, we observed that inhibiting hepatic SCD1 activity and subsequently changing the monounsaturated fatty acid (MUFA) to SFA ratios in tissues alters normal nutrient handling in skeletal muscle.

ACKNOWLEDGEMENTS

First and foremost I must thank with the utmost sincerity my advisor, Dr. Scott Harding, for his guidance, encouragement and friendship over the course of my graduate program. As my mentor and teacher, the skills and experiences I have acquired under his support are invaluable as they will be instrumental in future endeavours throughout my life. Thank you to my committee members, Dr. Jon McGavock, Dr. Miyoung Suh and Dr. Peter Jones, for their input, advice and thought-provoking discussions surrounding my thesis project. Thank you to NSERC and Merck & Co. for funding this project.

Thank you to the support staff from Human Nutritional Sciences at the University of Manitoba for considerable administrative assistance. A big thank you to fellow friends at the Richardson Centre for Functional Foods and Nutraceuticals who helped make this project fun and memorable. In particular, I must thank fellow lab technicians turned life-long friends, Maggie Wilson, Justine Reyes and Nicholas Winters, for all their help from learning how to pipette to reading introductions and making Western blotting something to look forward to. With their company in the lab, many laughs and memories were had to make this degree and experience one of the most rewarding of my life.

Finally, I sincerely thank my entire family for all their love and support throughout this tumultuous journey. To my husband, Jason, my rock, thank you for your constant love, patience and encouragement. To my sister, who continues to be a strong inspiration in my life. To my mother, thank you for your endless support, motivation and always being there for me. To my father, who instilled the virtue of perseverance, ambition, the pursuit of knowledge and who told me to never stop dreaming, this thesis is dedicated to you.

This thesis is dedicated to my father,

Larry K. Brezinski (1949-2009)

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

4EBP1	4E binding protein 1
ACC	acetyl-coA carboxylase
AIN	American Institute of Nutrition
AIN-93G	AIN-93 purified rodent diet for growth
AIN-93G-MX	AIN-93G mineral mix
AIN-93G-VX	AIN-93G vitamin mix
ALT	alanine aminotransferase
AMPK	adenosine monophosphate-activated protein kinase
ANOVA	analysis of variance
ASO	anti-sense oligonucleotide
AST	aspartate aminotransferase
ATP	adenosine triphosphate
β3-AR	β3-adregenic receptor
BAT	brown adipose tissue
BMC	bone mineral content
BSA	bovine serum albumin
CD36	fatty acid translocase/CD36
CE	cholesteryl ester
ChREBP	carbohydrate responsive element binding protein
Con	control
CPT	carnitine palmitoyltransferase
DDH_2O	deionized distilled water

DEXA	dual energy x-ray absorptiometry
DIO	diet-induced obesity
DNL	*de novo lipogenesis*
eIF4E	eukaryotic initiation factor 4E
ELOVL6	ELOVL fatty acid elongase 6
F	fasted
FA-CoA	fatty acyl coA
FAME	fatty acid methyl ester
FAS	fatty acid synthase
FFA	free fatty acid
GC	gas chromatography
GKO	global knockout
HC	high carbohydrate
HCVLF	high carbohydrate very-low fat
HDL	high density lipoprotein
HF	high fat
HRP	horseradish peroxidase
HTGL	hepatic triglyceride lipase
IMTG	intramuscular triglyceride
IR	insulin receptor
IRS1	insulin receptor substrate 1
JNK	c-JUN N-terminal kinase
LBM	lean body mass

LCn-3PUFA	long-chain omega-3 polyunsaturated fatty acid
LDL	low density lipoprotein
LKO	liver knockout
Lpl	lipoprotein lipase
MetS	metabolic syndrome
MI	myocardial infarction
mTOR	mammalian target of rapamycin
MUFA	monounsaturated fatty acid
NADH	nicotinamide adenine dinucleotide
NF	non-fasted
NS	not significant
PI-3K	phosphoinositide 3 kinase
PIP_2	phosphatidylinositol (4,5)-biphosphate
PIP_3	phosphatidylinositol (3,4,5)-triphosphate
PKB	protein kinase B
PKC	protein kinase C
PMSF	phenylmethylsulfonyl fluoride
PTP-1B	protein tyrosine phosphatase 1B
PUFA	polyunsaturated fatty acid
PVDF	polyvinylidene difluoride
Rheb	Ras homolog enriched in brain
RIPA	radioimmunoprecipitation assay
S6K	S6 kinase

SCD1	stearoyl-coA desaturase 1
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SE	standard error
SEM	standard error of mean
Ser^{65}	Phosphorylation at serine residue 65
Ser^{209}	Phosphorylation at serine residue 209
Ser^{307}	Phosphorylation at serine residue 307
Ser^{473}	Phosphorylation at serine residue 473
Ser^{2448}	Phosphorylation at serine residue 2448
SFA	saturated fatty acid
SPT	serine palmitoyl transferase
SREBP1c	sterol responsive binding protein 1c
TBHQ	*tert*-butylhydroquinone
TBS	tris-buffered saline
TBS/T	TBS/Tween-20
TC	total cholesterol
TG	triglyceride
Thr^{389}	Phosphorylation at threonine residue 389
TLR4	toll-like receptor 4
TNF-α	tumor necrosis factor-α
TP	total protein
TOP	terminal oligopyrimidine tract

TOR	target of rapamycin
TSC	tuberous sclerosis complex
UCP	uncoupling protein
VLDL	very-low density lipoprotein
VLF	very-low fat
VLF-M	very-low fat-monounsaturated group
VLF-S	very-low fat-saturated group
WAT	white adipose tissue

Chapter 1: LITERATURE REVIEW

1.1 Introduction

Obesity is a major public health concern with a staggering percentage of adults in Canada and the US classified overweight and obese. Obesity is a risk factor for a range of chronic health diseases including diabetes, cardiovascular disease and cancer, all of which are among the top causes of non-accidental death in North America. Until recently, obesity was generally a disease found only in developed countries; however the prevalence of obesity in developing countries has risen significantly in the past decade (World Health Organization; WHO, 2006). Most alarmingly, the proportion of children afflicted with obesity and diabetes is increasing worldwide, a trend that will increase the development of chronic disease as this population ages. Thus, there is a need for innovative approaches with which to prevent and/or manage obesity in our population.

Simplistically, the development of obesity is caused by an imbalance between energy intake and output but preventing and/or reversing obesity involves addressing a combination of genetic, environmental and metabolic factors. One of the major challenges in treating and reversing obesity lies in the dysregulation of numerous metabolic pathways which directly affect the reversal of the condition and because these pathways become altered they can accelerate the development of the comorbidities associated with obesity. Therefore, identifying and understanding the altered response to nutrient intake by key metabolic targets and the biological impact to the individual will contribute to the understanding of the pathogenesis and etiology of obesity and its comorbidities.

Investigating the elements which regulate *de novo* lipogenesis (DNL) is important to understanding the development of obesity. Because of its contribution to adiposity, the process of DNL is currently a research target for pharmacological treatments of obesity. The direct contribution of triglyceride (TG) production from DNL to the development of obesity is very small compared to that of dietary TG from chylomicrons. However, changes in normal hepatic DNL and TG production through metabolic intervention may alter both lipoprotein assembly and peripheral delipidation, indirectly giving DNL a greater role in the development of obesity (Timlin & Parks, 2005). The question then becomes what, if any, genes and enzymes that regulate fatty acid synthesis should be altered or inhibited in search of potential anti-obesity targets? Moreover, changes to hepatic DNL may also affect lipid metabolism in other tissues important in regulating adiposity, such as energy expenditure (i.e. skeletal muscle). Therefore, studying the effects on surrounding tissues and systems is of equivalent concern, primarily when single components of whole-body lipid metabolism are interrupted.

The mammalian stearoyl-coA desaturase-1 (SCD1) is purported to be an important substrate in the onset of obesity and its related comorbidities and serves as a key locus in lipid metabolism research as it is the rate-limiting enzyme in the synthesis of monounsaturated fatty acids (MUFA) from saturated fatty acids (SFA). Inhibited SCD1 activity will reduce DNL and increase energy expenditure through an increase in fat oxidation, two outcomes which potentially have serious implications in reducing diet-induced adiposity (Miyazaki *et al*., 2001).

1.2 Stearoyl-coA desaturase-1 (SCD1)

SCD1 is a fatty acid desaturase enzyme that catalyzes preferred substrates palmitic acid (16:0) and stearic acid (18:0) into products palmitoleate (16:1) and oleate (18:1) by introducing a single double bond at carbon-9 together with cofactors nicotinamide adenine dinucleotide (NADH), cytochrome b_5 reductase and cytochrome b_5. The products palmitoleate and oleate are the major MUFAs in TGs, phospholipids, cholesterol esters and wax esters and are preferred substrates for TG synthesis and cholesterol esterification. The saturated-to-unsaturated fatty acid ratio has been reported to regulate membrane fluidity and signal transduction influencing cell growth and neuronal differentiation and an alteration in this balance of fatty acids has been linked to various diseases including obesity, diabetes, cancer and cardiovascular disease (Storlien *et al.*, 1991; Jones *et al.*, 1996; Pan *et al.*, 1994; Pettegrew *et al.*, 2001; Solans *et al.*, 2000; Agatha *et al.*, 2001).

SCD is expressed in 4 gene isoforms (SCD1-4) in mice. There are 2 known SCD gene isoforms (SCD1 and -5) in the human genome, where human SCD1 shows 85% homology to murine SCD1 (Zhang *et al.*, 1999). SCD1 is expressed in an array of tissues including white and brown adipose tissue, skin, sebaceous glands and is expressed to a very high level in the liver when fed a lipogenic, high carbohydrate (HC) diet (Dobrzyn *et* al., 2010; Strabble *et* al., 2010). The remaining 3 murine isoforms, SCD2, SCD3 and SCD4, are mainly expressed only in the brain, Harderian gland, and heart, respectively. The expression of SCD1 is moderated by a multitude of processes, including dietary factors (glucose, cholesterol, and polyunsaturated fatty acids (PUFA)), environmental

factors (alcohol, metals, climate change, and nuclear receptor ligands), hormones (insulin, glucagon), developmental processes and peroxisomal proliferators (Flowers *et al*., 2009).

1.2.1 SCD1 activity and obesity

The action of SCD1 is a critical step in lipid metabolism and there is emerging evidence implicating increased SCD1 activity in the development of obesity, cardiovascular disease and the metabolic syndrome (MetS), but data from the human gene association studies and animal models are inconsistent (Stefan *et al*., 2008; Warensjo *et al*., 2007). Studies using mice with a natural SCD1 gene mutation (*asebia* ab^{J} and ab^{2J}) and mice with targeted knockout of the SCD1 gene ($SCD1^{-/-}$) have demonstrated that SCD1 plays a pivotal role in weight management and lipid partitioning. In particular, Ntambi et al demonstrated that the deletion of SCD1was anti-obesogenic via reduced DNL, reduced diet-induced adiposity and hepatic steatosis in mice fed a HC or high fat (HF) diet (Jiang *et al*., 2005; Miyazaki *et al*., 2001; Miyazaki *et al*., 2007; Ntambi *et al*., 2002). Using two models of SCD1 inhibition; global knockout (GKO) and tissue-specific liver knockout model (LKO), Ntambi and colleagues studied the metabolic effects of feeding chow, HF or HC diets. It was shown that GKO mice were protected from diet-induced obesity (DIO) regardless of diet, had reduced liver and plasma TGs but developed alopecia and eye fissures (Miyazaki *et al.*, 2007). Comparatively, the LKO SCD1 model was protected from the negative aspects of the GKO phenotype yet also showed resistance to HC DIO, reduction in DNL and exhibited increased insulin sensitivity (Miyazaki *et al.*, 2007). This deficiency in SCD1 activity results in an increase in energy expenditure, increased insulin sensitivity, decreased hepatic TG

synthesis, and decreased body adiposity causing them to be considerably leaner than their wild-type littermates (Ntambi *et al*., 2002; Dobrzyn *et al*., 2004; Gutierrez-Juarez *et al*., 2006; Miyazaki *et al*., 2007). However, the LKO model is not protected from weight gain when fed a lard-based HF diet as these mice gained weight to the same degree as their SCD1 flox/flox (Lox) littermates, deducing that this model is still susceptible to DIO under HF feeding, due to sufficient oleate provided within the HF diet (Miyazaki *et al*., 2007). This observation suggests that hepatic SCD1 function is necessary for normal DNL when fed a diet high in carbohydrates, although when fed a HF diet, extrahepatic SCD1 function is required and thus a deficiency in SCD1 function in these tissues would condone resistance to weight gain when fed the respective diets. The mechanisms responsible for the resistance towards HF and HC DIO in mice with global deletion of SCD1 are proposed to be due to an increased activation of lipid oxidation genes and reduced activation of lipid synthesis genes leading to the increase in energy expenditure (Miyazaki *et al*., 2007). Additional mechanisms responsible for the resistance to weight gain in GKO mice, particularly when fed fat-rich diets, points to the role SCD1 has in extrahepatic tissues such as adipose and skin, as well as lipid membranes. In a study by Jiang et al, mice with anti-sense oligonucleotide (ASO)-inhibition of SCD1 in liver and adipose tissue showed resistance to HF DIO while also exhibiting protection from skin disorders (Jiang *et al*., 2005). This protection originates from a reduction in the expression of multiple lipogenic genes in liver and fatty acid synthase (FAS) in white adipose tissue, that which the latter has been suggested to be a secondary observation of inhibiting SCD1 function in liver by ASO treatment. Nevertheless, inhibition of extrahepatic SCD1 without skin abnormalities indicates that ASOs may inhibit SCD1

without altering skin, hair and sebaceous gland function and histology (Jiang *et al*., 2005). Alternatively, disruption in the epidermal lipid barrier through a lack of SCD1 has been suggested to be a factor in the resistance to HF DIO through transepidermal water loss, disturbed thermoregulation, cold resistance and metabolic wasting (Binczek *et al*., 2007). Conversely, the HC DIO protection exhibited by SCD1 LKO mice is proposed to be due to an impairment of DNL originating from a reduction in carbohydrate sensitive gene expression, such as sterol responsive element binding protein-1c (SREBP-1c) and carbohydrate responsive element binding protein (ChREBP) and their downstream target genes directly involved in DNL prior to SCD1, including acetyl co-A carboxylase 1 (ACC1), FAS and ELOVL fatty acid elongase 6 (ELOVL6). This downregulation of fatty acid synthesis genes, primarily transcription factor SREBP, can normally be restored by refeeding with dietary MUFA, or oleate, but not SFAs, indicating that the induction of these lipogenic genes is reliant on dietary or endogenous oleate for normal lipogenesis (Sampath *et al*., 2007). SCD1-deficient mice also resisted the development of hepatic steatosis when fed a HF or HC diet and these effects were also shown *in vivo* in wild-type mice using an ASO inhibition of SCD1 (Brown *et al*., 2008). In addition, models using either SCD1 inhibition or targeted disruption of the SCD1 gene have exhibited increased fatty acid oxidation, energy expenditure and glucose clearance with simultaneous decreases in DNL, body adiposity and circulating insulin levels (Jiang *et al*., 2005; Miyazaki *et al*., 2001). These effects on lipid and glucose metabolism provide an insight into the metabolic consequences of dysregulating such a complex and highly regulated biochemical pathway, and look to question whether additional metabolic pathways such as skeletal muscle protein metabolism are altered as a result.

1.2.2 SCD1 activity and energy expenditure

Studies using SCD1 GKO mice and targeted disruption of SCD1 report a significant increase in energy expenditure contributing to the resistance to a HF or HC DIO and this observation is postulated to be due to various metabolic mechanisms but still remains largely unknown. One explanation for the increase in energy expenditure in response to SCD1 inhibition is constitutive activation of adenosine monophosphate-activated protein kinase (AMPK), which then triggers beta oxidation and depresses lipid synthesis by a series of phosphorylation and deactivation events (Dobrzyn *et al*., 2004). However, it has been shown that AMPK activation does not occur in SCD1 LKO mice that also exhibit increased energy expenditure, indicating that extrahepatic SCD1 deficiency is required for phosphorylation and activation of AMPK. Therefore, this upregulation of lipid oxidative genes and downregulation of lipid synthesis genes stemming from AMPK activation may be partially responsible for the increase in hepatic energy expenditure in the SCD1 GKO model. However, overall oxygen consumption is increased in all models suggesting that this increase in fat oxidation is originating from a myriad of sources which as of recent, research in this area of SCD1 inhibition is lacking. In addition, it has been shown that the increase in energy expenditure seen in SCD1 ASO-treated mice may also be attributed to an increased expression of uncoupling proteins (UCPs) 1-3 and β3-adregenic receptors (β3-ARs) in brown adipose tissue (BAT) causing an increased basal thermogenesis in these mice (Jiang *et al*., 2005). Also, in SCD1 GKO mice, UCPs are also increased in liver (*Ucp*-2), skeletal muscle (*Ucp-2* and *Ucp*-3) and white adipose tissue (WAT; *Ucp1*-3) (Rahman *et al*., 2005). This hypothesis is supported by results displaying lowered levels of hepatic adenosine triphosphate (ATP)

production due to the increased in these UCPs, which are thought to be shifted towards heat production rather than ATP synthesis.

1.3 Protein synthesis and energy expenditure

Protein synthesis is a metabolic process which is energy dependant, and ranges from peptide synthesis to complex polypeptides, encompassing everything from enzymes to skeletal muscle mass. Protein synthesis and cellular anabolism is the resultant product of mRNA translation initiation and subsequent protein synthesis and an increase in this process would demand an increase in energy, potentially requiring processes such as glycogenolysis and β-oxidation to be implemented in order to supply the necessary elements to sustain increased protein anabolism. Activation of the mammalian target of rapamycin (mTOR), a multifaceted protein kinase that regulates cellular growth has been studied in SCD-deficient mice for its proposed role in insulin signaling and observed increase in insulin sensitivity in these models but has yet to be studied in its relation to protein translation and anabolism as a possible link to the increase in energy expenditure.

1.3.1 mTOR and control of protein synthesis

mTOR is a serine/threonine kinase that is at the crux of a complex network of upstream and downstream signalling involved in a multitude of cellular activities ranging from protein transcription and translation to cytoskeletal organization (Jacinto *et al*., 2004). Activation of this protein kinase is largely regulated by nutrients and hormones, including glucose, fatty acids, amino acids and insulin. Any fluctuation in the handling

of these nutrients may have a serious effect on the productivity of this pathway, most importantly whether protein translation is blunted or stimulated.

Rapamycin is a compound originally isolated from soil samples on the South Pacific island of Rapa Nui, where it was identified to possess immunosuppressive and anti-tumor properties and later classified as a drug (Foster *et al*., 2010). This drug targets the protein suitably named TOR (target of rapamycin), which was originally identified in *Saccharomyces cerevisiae*, a unicellular budding yeast, that along with all other prokaryotes encompass two homologous TOR genes, TOR1 and TOR2, of which the cellular activities are shared (Heitman *et al*., 1991; Martin *et al*., 2005). Conversely, eukaryotes only possess one TOR gene but seem to have two similar complexes where one complex, mTORC1, is responsible for translation, cell size and growth, transcription, autophagy, ribosome biogenesis and protein stability, whereas the second complex, mTORC2, controls the cytoskeleton organization (Loewith *et al*., 2002, Kubica *et al*., 2008). mTORC1 is of specific interest as it is the complex that is rapamycin sensitive, regulates protein translation and is found to be activated by nutrients such as fatty acids, growth factors including insulin and cellular energy. mTORC1 is known to regulate mRNA translation by activating two downstream proteins, ribosomal protein S6 kinase 1 (S6K1) and eukaryotic initiation factor (eIF) 4E-binding protein-1 (4EBP1) bound complex. The eIF4E·4E-BP1 complex remains inactive until phosphorylated by mTORC1 where the bound proteins are released and eIF4E is free to form a complex with eIF4G, which then stimulates the initiation stage of mRNA translation (Hay *et al*., 2004).

At the cellular membrane level, the entire mTOR pathway is stimulated by the

presence of glucose, fatty acids, insulin and energy sufficiency, however the effect of varied levels on the regulation and activation on downstream signalling proteins and final protein synthesis is not well known. Given that nutrients and hormones play a large role in the activation of this pathway, irregularities in any of these stimulants may create a reactive effect on the signalling network and resultant protein translation (Davis *et al*., 2002; O'Connor *et al*., 2003).

1.3.2 Activation of mTOR

Insulin is the most complex and understood downstream mTOR activator and begins with an influx of the hormone through the basal membrane into the intracellular space where the insulin-receptor substrate 1 (IRS1) is phosphorylated via tyrosine and activates phosphoinositide-3-kinase (PI-3K). The activation of PI-3K launches the phosphorylation of the membrane lipid phosphatidylinositol-4, 5-biphosphate (PIP_2) creating the second messenger phosphatidylinositol-3,4,5-triphosphate (PIP_3). The presence of PIP_3 stimulates phosphoinositide-dependent kinase (PDK1) to combine with and phosphorylate Akt/PKB at Thr^{308} (protein kinase B) to bind as a complex to PIP_3. The activation of Akt/PKB phosphorylates the tuberous sclerosis protein (TSC) at Ser^{939} and Thr^{1462} inactivating the complex and moving downstream to increase Rheb (Ras homolog enriched in brain), a ras-like small guanosine triphosphate, which interrupts the action of FKBP38, an endogenous inhibitor of mTOR (Saltiel *et al*., 2001; Mothe-Satney *et al*., 2004; Hinault *et al*., 2006).

With the activation of mTOR, the same downstream effectors are in place regardless of cellular stimulant whereby two main proteins, S6K1 and 4EBP1 are

activated leading to mRNA translation. Initially, under non-stimulated conditions S6K and 4EBP1 are bound together to a control protein, eIF4E, where they are rendered inactive until dissociated from eIF3 through mTOR-mediated phosphorylation, Thr^{389} of S6K and Thr^{37} and Thr^{41} of 4EBP1. Once dissociated and active, S6K continues to phosphorylate ribosomal protein S6 where it contributes to direct regulation of mRNA containing a 5'- terminal oligopyrimidine tract (TOP) and eventual protein translation. Alternately, eIF4E, a cap-binding translation factor which binds to other translation factors such as eIF4G, together enable the assembly of initiation factors required in cap-dependent translation, is repressed by a 4EBP1·eIF4E bound complex. Once 4EBP1 is phosphorylated at Thr^{37} and Thr^{41} by mTOR, its affinity for eIF4E is reduced leading to the dissociation of the bound complex and allowing eIF4E to bind with eIF4G necessary for cap-dependent translation. Therefore, through mTOR-mediated phosphorylation of S6K and 4EBP1, protein synthesis is regulated via mRNA translation and cap-dependent translation through this cellular process (Hara *et al*., 1998; Shah *et al*., 2000; Proud *et al*., 2004).

It is well known that with sufficient supply of amino acids protein synthesis is triggered and maintained making amino acids the most potent activator of the mTOR pathway (Tremblay *et al*., 2001; Corradetti *et al*., 2006). Unlike insulin, amino acids, fatty acids and glucose are nutrient regulators of the mTOR signalling network and its mechanism of activation are much less complex but less understood. There are several competing hypotheses proposed for the amino acid-mediated mTOR activation however the most accepted model suggests that amino acids operate through a class 3 PI-3K

located upstream of mTOR and independent of all insulin signalling mechanisms (Nobukuni *et al*., 2005).

Comparatively, glucose is thought to activate mTOR by increasing ATP levels which subsequently inhibit AMPK leading to an inactivation of TSC where it then follows the insulin-mediated downstream pathway to activate mTOR (Fujii *et al*., 2006; Hardie *et al*., 2003; Hardie *et al*., 2006, Jeyapalan *et al*., 2007). Therefore, in a hyperglycaemic event, an immediate influx of glucose would create an overstimulation of mTOR leading to upregulation in mRNA translation and protein synthesis. Most interestingly, this pathway is significantly manipulated in the obese situation through a variety of stimulants including adipocytokines, free fatty acids (FFAs) and tumor necrosis factor-α that are conditionally increased and act to inhibit IRS1contributing to insulin resistance and downregulate mTOR activation and subsequent protein synthesis. Obese individuals demonstrate a decrease in adiponectin, a protein hormone that regulates fatty acid catabolism and glucose flux, and acts to reduce GLUT4 content creating a hyperglycemic event. Simultaneously, due to the increased adiposity, an increase in FFAs activates c-JUN N-terminal kinase (JNK) and inhibitor of nuclear factor κB kinase β (IKK), which together lead to serine phosphorylation of IRS1 and inhibition of IRS1 activation and decreased insulin sensitivity. In addition to modulation of adiponectin and FFA levels in the obese condition, tumor necrosis factor α (TNFα) is also activated, which triggers mTOR/p70S6k1 activation as well as protein kinase C (PKC), which together also lead to serine phosphorylated-IRS1 inhibition. Therefore, it is clear that elements modified through obesity can significantly contribute to the development of insulin resistance through their role in this extensive cell signaling pathway, however it is

still unclear how these elements, particularly fatty acids, contribute to mRNA translation and protein synthesis.

Although it has yet to be elucidated as to how fatty acids directly influence mRNA translation through the mTOR/S6K1 signaling network, it has been demonstrated that long-chain omega-3 polyunsaturated fatty acids (LCn-3PUFAs) incorporation into muscle membrane phospholipids play a prominent role in enhancing skeletal muscle insulin sensitivity and glucose mobilization. This improvement in insulin sensitivity promoted protein anabolism through increased mRNA translation and reduced whole-body amino acid oxidation to allow sufficient amino acid availability to warrant anabolism (Gingras *et al*., 2007). Another study investigating the role of LCn-3PUFAs of muscle phospholipids in protein metabolism using neonatal piglets exhibited a blunt in the typical developmental decline in the insulin sensitivity of amino acid disposal for protein anabolism compared to control piglets (Bergeron *et al*., 2007). In addition to the increase in insulin sensitivity demonstrated in LCn-3PUFA fed piglets, whole-body amino acid oxidation was reduced, permitting 41% more amino acids to accrete into proteins. Although insulin sensitivity was increased and amino acid oxidation was decreased, the fractional protein synthesis rate did not change by LCn-3PUFA feeding concluding that these fatty acids do not speed up the rate of protein anabolism but increase the efficiency at which it is produced by creating a state that favors anabolism over catabolism. Moreover, these long chain fatty acids are preferentially incorporated into membranes over other fatty acids, which act to modify the structure and function of the membrane as well as imparting a greater influence on cellular signal transduction and metabolic pathways over storage and adiposity. Therefore, the presence and level of fatty

acids may not have as much of an effect on the activation of this pathway as that of the type of fatty acids, particularly the length and saturation of the fatty acid, which may stimulate or blunt the phosphorylation and initiation of cofactors in this signaling cascade ultimately influencing protein translation.

Since this complex signalling pathway is entirely regulated by factors originating from or within the diet, the levels of these stimulants are constantly shifting leading to highly variable effects on mTOR activation. Due to the immense importance of an efficient mTOR signalling network on the cellular processes it is responsible for, a look into how these nutrients and hormones can affect the signalling pathway by downregulating or stimulating its activation is required.

mTOR activation is dependent on the phosphorylation of a series of upstream proteins which are initially activated by a number of growth factors including insulin, amino acids, lipids and cellular energy status (Jeyapalan *et al*., 2007; Kim *et al*., 2009). It is possible that dysregulation of these stimuli due to a metabolic condition may alter the expression of translation initiation factors within the mTOR pathway modifying all related processes, particularly protein synthesis and insulin-stimulated signal transduction and sensitivity. It is evident that the inhibition of IRS1 by serine phosphorylation is responsible for the potential mTOR-mediated insulin resistance by preventing the action of insulin leading to hyperinsulinemia, of which the majority of dysregulated growth factors have effect.

It is well known that the primary sites for postprandial glucose disposal are adipose and skeletal muscle in which efficient insulin signal transduction is necessary for proper glucose uptake. Defects in insulin-stimulated signal transduction in the skeletal

muscle are thought to contribute to the development of insulin resistance along with HF/HC (hypercaloric) diets (Reynolds *et al*., 2009). HF/HC diets combined with a defective insulin signaling cascade may contribute directly to insulin resistance, a pathophysiology that precedes the onset of type 2 diabetes. Recent studies have shown that the progress of insulin resistance is mediated through the activation of the mTOR signaling network via a negative feedback mechanism through serine phosphorylation and subsequent inhibition of IRS1, the protein responsible for insulin-mediated activation of mTOR (Khamzina *et al*., 2005).

In the case of obesity, the constant influx of FFAs inhibits IRS1 activation and the reduced level of adiponectin decreases the expression of GLUT4 resulting in hyperglycemia, which also inhibits IRS1 activity by way of the atypical protein kinase C ε and δ. In addition, sustained activation of mTOR and downstream p70S6K leads to the phosphorylation of IRS1 on numerous inhibitory loci leading to its degradation and blunted response to insulin through a negative-feedback loop. Moreover, the deleterious action on IRS1 by obesity and type 2 diabetes also impacts mRNA translation and protein synthesis through downregulation of total mTOR activity and a reduction in all associated processes. For instance, obesity causes constitutive activation of mTOR/p70S6K leading to IRS1 degradation and subsequent impairment of PI-3K and mTOR activation. Reduced mTOR activity would translate into reduced activation of downstream proteins responsible for mRNA translation and eventually developing into impaired protein synthesis in skeletal muscle. Interestingly, with SCD1 inhibition insulin sensitivity is improved and hyperglycemia is reduced. However, if this improved insulin sensitivity is derived from liver or adipose cells and not skeletal muscle cells, impairment in protein

synthesis may still occur due to the insulin-mediated mTOR activation required in skeletal muscle for mRNA translation (Chevalier *et al*., 2005). Comparatively, it is has been shown that mTOR inhibition promotes β-oxidation of exogenous fatty acids, attenuates re-esterification of FFAs and DNL and essentially improves the metabolic condition through increased energy expenditure (Reynolds *et al*., 2009).

1.3.3 Influence on insulin resistance and the mTOR/S6K1 signaling network from SCD1 inhibition

As previously mentioned, the mTOR signaling network has only been studied in its relation to insulin sensitivity in SCD1-deficient models. It is understood that many aspects regarding cell signaling will be modulated in response to SCD1 inhibition as the saturation composition of membrane phospholipids is modified from oleate, an unsaturated lipid to one that is fully saturated, stearate. This change in saturation of membrane phospholipids may have a significant effect on the inner workings of the Akt/mTOR/S6K1 pathway and its products influencing insulin sensitivity and possible protein translation. In a study by Rahman and colleagues, $SCD1^{-/-}$ mice demonstrate increased insulin signaling in muscle via increased tyrosine phosphorylation of both IR and IRS1 and IRS2, increased phosphorylation of Akt and downregulated expression and activity of protein tyrosine phosphatase 1B (PTP-1B) (Rahman *et al*., 2003). These observations were proposed as a link to the increase in insulin sensitivity associated with a loss in SCD1 function. The central implication of an increase in IRS1 and Akt phosphorylation is their downstream effect on GLUT4 translocation and subsequent glucose uptake and thus, an increase in the activity of these cofactors signals an increase

in GLUT4 translocation to the plasma membrane to facilitate an increase in glucose uptake signaling an increase in insulin sensitivity. Conversely, PTP-1B is a protein phosphatase whose activity downregulates insulin signaling through dephosphorylation of insulin receptor (IR) and diminishes the insulin response, which was demonstrated in PTP-1B$^{-/-}$ mice that displayed a prolonged insulin response due to constant phosphorylation of IR when compared to PTP-1B$^{+/+}$ mice. According to the study by Rahman et al., a loss of SCD1 function triggers a reduction in mRNA levels, protein mass and activity of PTP-1B in addition to upregulated activity of IR, IRS1 and IRS2 proteins, collectively increasing the response to insulin upon a glucose load (Rahman *et al*., 2003). The mechanism originating from SCD1 deficiency that leads to these changes in insulin signaling is still under investigation but points to links in relation to intramuscular lipid accumulation, FFAs and ceramide levels.

1.4 Nutrient handling and the influence on mTOR activation from SCD1 inhibition

The activation of mTOR relies on the phosphorylation of numerous upstream target proteins which as previously discussed are triggered through a variety of stimulants, of which dietary nutrients are primary actuators alongside hormones like insulin and glucagon. Regardless of what individual nutrient or hormone is triggering this pathway, they all stem from the diet, and along with alternate metabolic pathways such as DNL, the height of their activity occurs postprandially.

In the postprandial state, dietary nutrients endure many metabolic processes to catalyze them from macronutrients to microelements that are capable of entering the cell to stimulate activation of upstream target proteins of the mTOR pathway. Multiple

lipolytic, glycolytic and transferase enzymes are responsible for the catalysis of these dietary nutrients and the functional efficiency of these catalytic enzymes may be largely regulated through the genetic framework. Thus, the activity of these enzymes may vary drastically in response to individual expression of these genes, creating an individual effect on metabolic processes such as DNL, cholesterol synthesis and protein synthesis via mTOR activation.

Furthermore, the effect that pharmacological interventions may have on these normal, frequent processes may be detrimental and cause significant whole body effects, possible contributing to excess adiposity, obesity and MetS or can be beneficial and serve as protection to these diseases and investigating the effect that SCD1 inhibition has on these enzymes and processes may provide an insight into this question.

1.4.1 Effect of SCD1 inhibition on fatty acid and lipoprotein composition

The inhibition of SCD1 reduces DNL mainly by the inability to create the intended products oleate and palmitoleate, both MUFAs, however what is the fate of saturated substrates palmitoyl-CoA and stearoyl-CoA and how do they alter other fatty acid derived substances, such as cholesterol, lipoproteins and FFAs?

In a study by Brown et al, SCD1 was inhibited using ASOs in a mouse model of hyperlipidemia and atherosclerosis ($LDLr^{-/-}ApoB^{100/100}$) to investigate the role of SCD1 in atherosclerosis progression and lipoprotein metabolism. They revealed that in congruence with earlier reports, SCD1 inhibition protected against DIO, insulin resistance and hepatic steatosis but unexpectedly accelerated aortic atherosclerosis (Brown *et al*., 2008). In this study, it was demonstrated that inhibiting SCD1 creates a

significant accumulation of SFAs in TGs and cholesteryl esters (CEs) and this enrichment in SFAs is observed to stimulate the secretion of proinflammatory cytokines in macrophages through the activation of toll-like receptor 4 (TLR4), a mechanism whose activity is exacerbated when the macrophage lipids become saturated. Brown et al also observed that SCD1 inhibition had additional effects on plasma lipoprotein distribution and composition, where very-low density lipoprotein (VLDL) cholesterol and high density lipoprotein (HDL) cholesterol was reduced, whereas there was no change in low density lipoprotein (LDL) cholesterol distribution; however the degree of saturation of all lipoproteins was significantly modified. It was shown with SCD1 inhibition and significantly reduced MUFAs that the composition of low density lipoprotein-cholesteryl esters (LDL-CE) became considerably enriched with SFA and HDL-CE to a lesser extent. This enrichment of lipoproteins with SFA contributes to the increased proinflammatory response and aortic lesions as these SFA-rich LDL particles are responsible for the transport of these now SFAs to numerous tissue cells, including skeletal muscle, liver and heart and may contribute to the modification in functionality of these tissues. The effect that increased saturation of these lipoproteins has on other elements may be dramatic as eventually these SFAs may be incorporated to form saturated membrane phospholipids, macrophage lipids, membrane lipid bilayers potentially disrupting the normal equilibrium of these substances and affecting their role in important processes such as signal transduction and neuronal differentiation. Furthermore, modifying the composition of fatty acid-derived substances such as lipoproteins, TGs and CEs may alter the ability and efficiency of lipolytic, lipogenic and lipid transfer enzymes to function properly or may modify how these enzymes function completely due to the change in substance function

recognition. For example, it has been reported that HF diets enhance the activity of lipoprotein lipase (Lpl), a multifunctional enzyme responsible for hydrolyzing the TG core of lipoproteins, but most interestingly, unsaturated fats, particularly PUFA, heighten Lpl activity significantly more than do saturated fats, whereas hepatic triglyceride lipase (HTGL) responds in the reverse manner, where its activity is stimulated preferentially to saturated fats (Coiffier *et al*., 1987). For metabolic processes that rely on the fatty acid products from Lpl-mediated lipoprotein hydrolysis, a change in enzymatic activity could greatly affect downstream whole body processes, particularly lipid synthesis and storage or lipid oxidation and the predisposition to fat accumulation and obesity.

1.4.2 Role of lipolytic and lipotransferase enzymes in nutrient handling

Activity level of lipogenic and lipolytic enzymes are essential in determining endogenous lipid status, either fat storage or fat oxidation, greatly influencing body adiposity. In addition to lipid partitioning, the expression and activity level of these enzymes may affect non-lipid processes such as glucose metabolism, protein metabolism and their respective disorders; insulin resistance and muscle wasting.

For instance, in a study by Kim et al, it was demonstrated that an overexpression of Lpl in muscle caused muscle-specific insulin resistance in mice, whereas insulin sensitivity in liver remained unchanged (Kim *et al*., 2001). Furthermore, an accumulation of myocellular lipid was deposited in muscle in response to an overexpression in Lpl, suggesting an association between muscle-specific overexpression of Lpl and the rate of lipid deposition and development of insulin sensitivity in skeletal muscle (Kim *et al*., 2001). Alternatively, Wang and colleagues investigated the effects of

reduced muscle-specific Lpl activity on insulin sensitivity in both muscle and liver using a skeletal muscle-specific Lpl knockout mouse ($SMLPL^{-/-}$) and showed an improvement in insulin signaling while reducing lipid accumulation, both in skeletal muscle; however decreased insulin sensitivity was exhibited in liver in the $SMLPL^{-/-}$ model (Wang *et al*., 2009). These contrasting observations stem from the central function of Lpl as a lipoprotein-derived fatty acid partitioning "gatekeeper", where the activity level of this lipolytic enzyme fluctuates with nutrient status, where it is heightened in a fed state and reduced in a fasted state, but is also regulated by insulin and glucose levels in the same manner.

In addition to tissue-specific transcriptional, posttranscriptional and posttranslational regulation of Lpl, fluctuating nutrition and hormonal states impart escalated effects to Lpl activity causing divergent effects to lipid partitioning. These differential effects can either create a flood of Lpl-catalyzed reaction products, like FFAs and monoglycerols, to be oxidized for energy use or shuttled to adipose, macrophages, skeletal or cardiac muscle for storage as neutral lipids, CEs or TGs; or create an accumulation of TG-rich lipoproteins in the blood contributing to circulating cholesterol and TG levels (Wang & Eckel, 2009). These divergent effects that come from differing levels of Lpl activity appear to have significant effects on insulin signaling in skeletal muscle as previously mentioned in the study by Wang et al, which have been observed to be linked back to the intramuscular triglyceride (IMTG) content and the effect that an accumulation of IMTG has on insulin signaling cofactors (Wang *et al.*, 2009). For instance HF-fed $SMLPL^{-/-}$ mice were observed to have reduced IMTG compared to control which correlated to a significant increase in insulin-stimulated skeletal muscle

glucose uptake as well as insulin-stimulated glucose transport in soleus muscle. When cofactors within the insulin signaling cascade were investigated to determine whether the increase in glucose uptake was due to increased activation of IR, IRS-1 and Akt, it was demonstrated that the total levels of IR, IRS-1, Akt and the insulin-stimulated phosphorylation of IR and IRS-1 surprisingly remained unchanged in $SMLPL^{-/-}$. Although major determinants of insulin signaling were not changed, a two-fold increase in insulin-stimulated phosphorylation of Ser-473 Akt was demonstrated in $SMLPL^{-/-}$ when compared to control, while phosphorylation of Thr-308 Akt was also increased under both basal and insulin-stimulated conditions (Wang *et al*., 2009). This observation of reduced IMTG accumulation, increased glucose uptake, and increased phosphorylation of basal and insulin-stimulated Akt without increased activity of IR and IRS-1 led Wang et al to suggest that Lpl may regulate skeletal muscle Akt phosphorylation through its effects on other lipid derivatives, possibly having an effect on the phosphorylation of upstream lipid phosphatases PIP_2 and PIP_3 and subsequent Akt activation (Wang *et al*., 2009). Therefore, it is clear that the level of Lpl activity and thus, level of lipoprotein-derivatives available for oxidation or storage can dramatically affect cell signaling within many metabolic processes, particularly the insulin signaling network that regulates insulin sensitivity. As previously discussed, this insulin signaling network that regulates glucose uptake also plays a crucial role in mRNA translation through the activation of mTOR and S6K and thus fluctuating Lpl activity levels due to nutrient and hormone status may have a significant effect on resultant protein synthesis, however this effect remains to be evaluated.

Another enzymatic protein whose activity level may be influenced by nutrient status and may have an effect on the insulin signaling pathway is fatty acid translocase/CD36 (CD36), a highly glycosylated transport protein that acts to interact with and transport fatty acids across phospholipid bilayers to facilitate fatty acid oxidation together with mitochondrial carnitine palmitoyltransferase-1 (CPT1) (Holloway *et al*., 2006). This transport protein has been shown to induce translocation to the plasma membrane from the intracellular pool by insulin, proposed to involve PI-3K signaling, the protein that is activated by IRS-1 phosphorylation within the insulin signaling network, which acts in the same manner as GLUT4 translocation to facilitate glucose uptake (Holloway *et al*., 2008). The main role of CD36 lies within the fatty acid transport and oxidation process whereby the traditional view involves mitochondrial transport of fatty acyl-CoAs (FA-CoAs) mainly by CPT1 facilitated by CD36 translocase activity of these acyl-CoAs from the lumen into the interstitial fluid and again through the plasma membrane into the cellular space. Recent reports have shown that CD36 has a greater role in fatty acid transport and oxidation than previously thought demonstrating a role in mitochondrial transport of FA-CoAs in concert with CPT1, whereby CD36 is translocated to the mitochondria suggested to regulate fatty acid oxidation, the mechanisms of which are currently unknown (Holloway *et al*., 2008). Of particular interest are the differential effects that obese and non-obese states has on CD36 activity and thus, how these states alter fatty acid oxidation in skeletal muscle. For instance, it has been shown in both obese animals and humans that FA-CoA transport is increased along with an increase in plasma membrane CD36 levels; however, skeletal muscle fatty acid oxidation is attenuated (Han *et al*., 2007). This reduction in fatty acid oxidation

cannot be attributed to a decrease in transport or decreased FA-CoA concentrations, but is suggested to possibly be due to a shift of FA-CoA use from oxidation to esterification in the state of obesity or a reduced capacity for mitochondria to oxidize FA-CoAs; either by decreased mitochondrial content or malfunction within the mitochondrial fatty acid oxidation process itself (Holloway *et al*., 2008). Furthermore, it has been demonstrated that in the obese state, CD36 has an inability to translocate to the plasma membrane to facilitate fatty acid uptake in response to muscle contraction and thus may also display an inability to relocate to the mitochondria contributing to the reduction in fatty acid oxidation (Han *et al*., 2007).

1.5 Summary

With the inhibition of SCD1, normal DNL is interrupted preventing the production of MUFAs, oleate and palmitoleate, meanwhile causing SFA substrates, palmitoyl- and stearoyl-CoA, to remain in excess. The increased presence of hepatic saturated fatty acyl CoAs will be directed toward oxidation but will also result in TG synthesis, now with a higher SFA content. Eventually, increased assembly and secretion of VLDL particles will occur with higher levels of SFAs, all which is exacerbated during HC feeding. Under normal conditions, VLDL particles transport TGs to the adipose and muscle tissue via circulation, where they are hydrolyzed by Lpl in order to release the glycerol and fatty acids for oxidation to supply energy or be stored as TGs once re-esterified. However, the liver TG pool is increased during HF and/or HC feeding, resulting in an increase in the plasma TG pool via increased VLDL secretion. Insulin

resistance exacerbates this effect due to reduced efficiency of VLDL export of lipid into peripheral tissues, such as adipose and skeletal muscle. When SCD1 activity is inhibited the resulting increase in SFAs will suppress DNL in favour of hepatic oxidation. However, the excess of SFAs which are directed toward lipoproteins will be less efficiently packaged and hydrolyzed at the peripheral tissues because of the increased SFA content (Brown *et al*., 2008; Strabble *et al*., 2010).

The effects that this accumulation SFA-derivatives has on peripheral tissues, mainly skeletal muscle, and how these nutrients are handled, hydrolyzed and transported in these tissues are currently unknown and may have significant effects on metabolic processes that rely on these nutrients as stimulants, primarily processes that may regulate insulin sensitivity and modify energy expenditure, cell growth and protein translation.

Chapter 2: OBJECTIVES AND NULL HYPOTHESES

2.1 Study Proposal

While there are potentially positive outcomes following SCD1 inhibition (i.e. increased beta-oxidation, energy expenditure, insulin sensitivity, glycemia) the direct effect on peripheral tissues is still not well understood. As the main tissues for glucose disposal, increasing insulin sensitivity and beta-oxidation in adipose and skeletal muscle is essential when addressing insulin resistance. Therefore, investigating the effects on these tissues in response to SCD1 inhibition is crucial for the purpose of targeting effective anti-obesity and insulin resistance treatments. This study was designed to determine whether the disruption of normal DNL by SCD1 inhibition, with underlying diet-induced insulin resistance, will affect skeletal muscle metabolism. The SCD1 inhibitor used in this study is a thiazole amide analog that affects oleate production by reducing the 18:1/18:0 ratio contributing to endogenous oleate levels. A 10 week high carbohydrate very low fat (HCVLF) diet was used to create a model of diet-induced insulin resistance in C57BL6 wild-type mice. An oleate-sufficient diet (1.5% triolein, VLF-M) and a very low-oleate diet (1.5% tristearin, VLF-S) were used to determine the relationship between oleate supplied through the diet versus endogenously produced oleate and the effects on skeletal muscle metabolism. We studied the effects of changes in skeletal muscle protein translation signalling through the mTOR pathway in response to changes in endogenously produced oleate through SCD1 inhibition. This study also determined whether acute SCD1 inhibition effects skeletal muscle protein expression of glucose uptake regulators GLUT4 and lipid uptake regulators Lpl and CD36.

2.2 Objectives and Null Hypothesis

2.2.1 Objectives

This project is a sub-project from a larger study investigating the metabolic effects of inhibiting SCD1 in wild-type mice fed a HCVLF diet to induce insulin resistance. The primary goal of the main study was to determine the effects of SCD1 inhibition on cholesterol, fatty acid and glucose metabolism with specific changes in gluconeogenesis, lipogenesis and cholesterol synthesis in response to changes in endogenous and dietary levels of oleate.

The first objective was to determine whether short term SCD1 inhibition following a 10 week HCVLF impacts lipid and glucose metabolism by altering known biomarkers (blood TGs, cholesterol, glucose, etc.). To achieve this objective animals were administered the SCD1 inhibitor by gavage 72 hours prior to the end of the study, therefore the responses to SCD1 inhibition represent acute loss of SCD1 function.

The second objective was to establish changes in the phosphorylation of certain upstream and downstream proteins of the mTOR insulin signaling machinery in response to dietary treatments and SCD1 inhibition to determine effects on protein translation in skeletal muscle. To achieve this objective phosphorylated protein content was determined compared to total protein content of specific proteins within the mTOR pathway, namely IRS1, Akt, mTOR, S6K, 4EBP1 and eIF4E, therefore relative levels of phosphorylation represent activation of specific proteins and eventual protein translation.

The third objective was to examine whether SCD1 inhibition under HCVLF feeding altered the expression of specific nutrient handling proteins, Lpl, CD36 and GLUT4, within skeletal muscle to establish a biochemical origin for observed metabolic effects of SCD1 inhibition. To achieve this objective, levels of total skeletal muscle protein content of each molecular protein were assessed via immunoblotting and represent respective levels of activity from each particular nutrient handling protein.

The final objective was to examine the first three objectives on the basis of different energy states and different dietary treatments, specifically to compare the metabolic effects observed in response to SCD1 inhibition between non-fasted and fasted conditions and to compare effects between mice fed VLF-M and VLF-S diets. These conditions will determine to what extent any metabolic effects in response to SCD1 inhibition are exacerbated or attenuated.

2.2.2 Null Hypothesis

1. That, acute SCD1 inhibition in wild-type C57Bl6 mice will have no effect on plasma total cholesterol, non-HDL cholesterol, HDL cholesterol and TG concentrations.

2. That, acute SCD1 inhibition in wild-type C57Bl6 mice will have no effect on protein translation signalling via insulin-mediated mTOR activation.

3. That, acute SCD1 inhibition in wild-type C57Bl6 mice will not change the expression of specific nutrient handling proteins, GLUT4, Lpl and CD36, in skeletal muscle.

Chapter 3: MATERIALS & METHODS

3.1 Study Protocol

3.1.1 Experimental Animals

Male wild-type C57BL6 mice were purchased from Charles Rivers Laboratories (Montreal, QC). Prior to study commencement, mice were housed in pairs in a temperature-controlled room with a 12-hr light-dark cycle for a 2 week acclimatization period. During acclimatization, mice were provided *ad libitum* with standard rodent chow and water. All experimental animals were maintained in a Canadian Council on Animal Care-approved pathogen-free animal facility at the Richardson Centre for Functional Foods and Nutraceuticals. All animal procedures involved the humane care and use of the animals, were performed within the Canadian Council on Animal Care guidelines and approved by the Animal Ethics Board of the University of Manitoba. See Appendix 1-4 for Animal Care protocol, amendment and approval.

3.1.2 Study Design

This study was conducted in 2 phases, non-fasted and fasted using 128 wild-type C57BL6 mice. Each study phase was conducted over a 10 week period using 64 mice, which were approximately 5 weeks old and weighing an average of 20 grams at the beginning of the experiment. Following the 2 week acclimatization period, mice were randomized to one of two HCVLF dietary treatments containing either 1.5% triolein (VLF-M) or 1.5% tristearin (VLF-S) and were fed assigned treatment over a 10 week

period. Within each dietary treatment, mice were also randomized to receive a dose of either 0.1% methylcellulose with a small molecule inhibitor of SCD1 (SCD1-) or 0.1% methylcellulose (Con) alone during the last 3 days of the dietary treatment.

3.1.3 Study Diet

Study diets were purchased and prepared in consultation with Harlan Teklad (Madison, WI). The composition of each diet is outlined in Table 1. HCVLF diets contained a total of 2% fat (w/w; 5% kcal) of which 1.5% was either triolein (Harlan Teklad custom research diet, product #: TD08241; Appendix 5a) or tristearin (Harlan Teklad custom research diet product #: TD08242; Appendix 5b) and the remaining 0.5% total fat was safflower oil (0.4%) and flaxseed oil (0.1%). Triolein (oleate) was chosen to provide a dietary source of MUFAs, whereas tristearin (stearate) was chosen to provide a source of SFAs as the major fat component. Safflower oil and flaxseed oil were incorporated to supply essential fatty acids, linoleic and linolenic acid, respectively. Nutrient information is outlined in Table 2. The carbohydrate component of the diet was sucrose and comprised 68.7% of weight or 75% kcal. The protein source was casein and provided 17.4% of diet weight or 19.2% kcal. Non-nutritive food coloring was added (blue, pink) to distinguish each diet treatment.

Table 1. Experimental diet composition[1]

Ingredient	VLF-M	VLF-S
Casein, "Vitamin-Free" Test	190.0	190.0
DL-Methionine	3.0	3.0
Sucrose	500.0	500.0
Corn Starch	187.84	187.84
Glyceryl trioleate	15.0	
Glyceryl tristearate		15.0
Safflower, linoleic	4.0	4.0
Flaxseed Oil	1.0	1.0
Cellulose	50.0	50.0
Mineral Mix, AIN-93G-MX (94046)[2]	35.0	35.0
Calcium Phosphate, dibasic	1.5	1.5
Vitamin Mix, AIN-93G-VX (94047)[3]	10.0	10.0
Choline Bitartate	2.5	2.5
TBHQ, antioxidant[4]	0.01	0.01
Blue Food Color	0.15	
Pink Food Color		0.15

[1] Values are expressed as g/Kg
[2] AIN-93G mineral mix
[3] AIN-93G vitamin mix
[4] *tert* - Butylhydroquinone

Table 2. Experimental diet nutrient information[1]

	HCVLF[2]	
	% by weight	**% kcal from**
Protein	17.4	19.2
Carbohydrate	68.7	75.8
Fat	2.0	5.0
Kcal/g **3.6**		

[1] Values are calculated from ingredient analysis or manufacturer data
[2]Nutrient data are identical for VLF-M and VLF-S diets

3.1.4 SCD1 small molecule inhibitor

The SCD1 inhibitor used in this study was a thiazole amide analog, MF-438, developed by Merck Frosst Centre for Therapeutic Research, which has been shown to acutely and effectively suppress SCD1 activity at a dose of 3mg/kg body weight (Li *et al*., 2009; Leclerc *et al*., 2011). The chemical structure of MF-438 is displayed in Figure 1 (Leclerc *et al*., 2011). This treatment was administered in a 0.5% methylcellulose suspension and was dosed orally via gavage at the end of the dietary treatment 3 days prior to blood and tissue collection. The inhibitor was administered during the last 3 days of dietary treatment to establish an acute response to SCD1 inhibition and reduced DNL following a long term diet treatment.

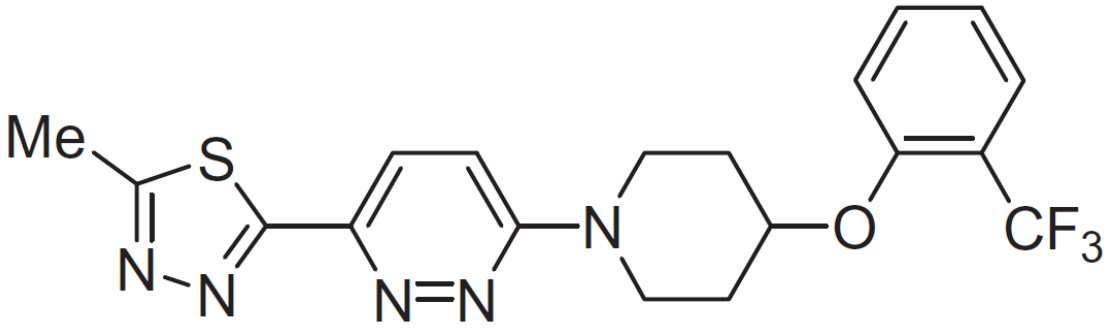


Figure 1. Chemical structure of SCD1 thiazole amide analog inhibitor, MF-438.
(Reproduced from Leclerc *et al*., 2011)

3.2 Data Collection

3.2.1 Body weight and food intake

Body weight was measured and recorded once per week over the 10 week study period at approximately the same time for each weighing. Study diets were administered and recorded two to three times per week depending on consumption. Food intake was determined by the sum of total food given less the amount remaining in the cage at the end of the study since the method of administration provided very minimal food loss to cage shavings. Diet consumption was confirmed by presence of respective blue or pink-colored (triolein or tristearin) feces.

3.2.2 Body composition assessment

Body composition was assessed on weeks 2 and 9 of study. Mice were anaesthetized via intraperitoneal injection of ketamine/xylazine (100/10 mg/kg body weight) and body composition was measured via dual energy x-ray absorptiometry (DEXA) (Lunar Prodigy Advance, GE Healthcare, Madison, WI, USA) prior to SCD1 inhibitor administration. Whole body scans were measured and data including total lean mass, total fat mass and total fat percentage was determined (Encore 2005, v.9.30.044, GE Healthcare, Madison, WI, USA).

3.2.3 Blood and tissue collection

Mice were anesthetized via isoflurane following a 12 hour fast for the fasted phase of the study, whereas for the non-fasted phase, food was not removed. Once mice

were in a surgical plane of anesthesia, blood was collected from mice via cardiac puncture into heparinized collection tubes. Blood samples were centrifuged at 3500 x g for 15 min at 4°C and plasma was aliquoted for storage at -80C for further analysis. Mice were killed by exsanguination and removal of the heart, followed by removal of the liver, epididymal adipose tissue and soleus muscle. Tissues were blotted on ice cold saline-soaked gauze, frozen by immersion in liquid nitrogen and stored at -80C. Biological samples from each pair housed cage were pooled for further analysis to provide a final sample size of 32 for each phase.

3.3 Endpoint Measurement Methodologies

3.3.1 Plasma biochemical profile

Plasma TG, total cholesterol (TC), glucose, direct high density lipoprotein (dHDL), total protein (TP), albumin and lipase levels were measured via a series of colormetric assays using the Ortho-Clinical Diagnostics Vitros®350 Chemistry System (Rochester, NY, USA). Non-HDL cholesterol values [LDL + VLDL + intermediate density lipoprotein (IDL)] were calculated by subtracting the TC measurement from the dHDL measurement. Plasma albumin and TP were measured as biomarkers of inflammation to indicate possible hepatic stress in response to SCD1 inhibition, as well as changes in protein synthesis. Circulating lipase levels were quantified and assessed to determine to what degree TG are being hydrolyzed in the endothelium following SCD1 inhibition in response to an overproduction of VLDL particles.

3.3.2 Plasma glucose and insulin assessment

Plasma glucose concentrations were determined using a glucose assay kit (Genzyme, Mississauga, Ontario, Canada) and plasma insulin concentrations were determined using a mouse insulin enzyme-linked immunosorbent assay kit (ELISA) (Millipore, Billerica, MA, USA). These methods determined whether blood glucose levels and insulin sensitivity was altered due to the disruption of SCD1.

3.3.3 Immunoblot analysis

3.3.3.1 Protein Extraction

Soleus skeletal muscle was extracted from mice following euthanasia and stored as previously described. 10X radioimmunoprecipitation assay (RIPA) lysis buffer was diluted to a 1X working solution with deionized distilled water (DDH_2O). 1mM phenylmethylsulfonyl fluoride (PMSF) solution was added to lysis buffer immediately prior to use. 100X Protease/Phosphatase inhibitor cocktail (Cell Signaling) was diluted to 1:100 in chilled 1X RIPA buffer immediately prior to homogenization. Tissues were measured to approximately 100mg and homogenized in 1ml of ice-cold RIPA buffer (Cell Signaling) for 30 seconds. Following homogenization, lyzed samples were left to incubate on ice for 5 minutes, and then sonicated for 5 minutes. Samples were then centrifuged for 10 minutes at 14,000g in a chilled microcentrifuge to separate extracted protein from cell debris. Extracted protein supernatant was aliquoted and stored at -80C for future use.

3.3.3.2 Protein Concentration Determination

Total protein concentration was determined using the Bradford method (Bradford *et al*., 1976), a colormetric protein assay. A series of protein standards were prepared using 0.2 mg/dl bovine serum albumin (BSA) diluted with DDH_2O to a final concentration of 0 (blank = DDH_2O only), 0.25, 0.5, 0.75, 1.0, 5, 10, 15, 20, 25 µg BSA/ml. 400 µl of each standard and 10 µl of each sample was added to separate test tubes where 390 µl of DDH_2O was added to sample tubes. 2 µl of Coomassie Blue was added to each test tube for a final volume of 402 µl. Each test tube was vortexed for 5 seconds and left to incubate for 10 minutes. Following incubation, standards and samples were transferred to clean spectrophotometer cuvettes and absorbance was read at a wavelength of 595nm using a Cary50 spectrophotometer (Cary, NC, USA). Absorbances of standards were plotted against concentration with a linear fit to produce a standard curve to which the concentrations of unknown samples were calculated.

3.3.3.3 Protein Denaturation

Thirty-five micrograms of total protein were diluted with DDH_2O to a volume of 100 ml and further diluted (1:1) with 2X gel loading buffer consisting of 0.5M Tris HCl (pH 6.8), 3.9% β-mercaptoethanol, 10% sodium dodecyl sulphate (SDS), 10% glycerol and 0.002% bromophenol blue. Loading samples with a final volume of 2ml were boiled at 100°C for 5 minutes and stored at -20°C for future use.

3.3.3.4 Protein electrophoresis and transfer

Thirty-five μg of total denatured protein were loaded into each lane and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 4-20% polyacrylamide resolving gel (Mini-Protean TGX Precast Gels, Bio-Rad, Missasauga, Ontario, Canada). Each gel consisted of each study treatment for a sample size of 3, where 4 gels were performed for each protein of interest to include all study samples, of which was performed in triplicate. A molecular weight ladder (Precision Plus; Bio-Rad) and an internal loading control were also included on each gel. Gel electrophoresis was performed using the Bio-Rad Mini-Protean Tetra Cell, which is a 4-gel system including electrode assembly, clamping frame, companion module, tank, lid with power cables and mini cell buffer dam (Bio-Rad). Gel electrophoresis was performed at 90V for approximately 30 minutes or until the samples had run through the stacking layer of the acrylamide gel. Voltage was increased to 200V and continued to run for approximately 1 hour or until the protein of interest had separated, all of which was performed while the tank was submerged in ice.

Following electrophoresis, proteins with a molecular weight less than 100kDa were transferred by semi-dry transfer onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-FL, Millipore, Billerica,MA, USA) at 20V for 45 minutes. Proteins with a molecular weight over 100kDa were transferred by wet transfer at 100V for 1 hour while submerged in ice. Prior to transfer, transfer cassette sandwich components were prepared for assembly; filter paper was saturated in transfer buffer solution consisting of Tris base, glycine and methanol and PVDF membrane was pre-soaked in methanol for 5 minutes and then rinsed in transfer buffer for 1 minute where it was then placed on top of the gel

with the filter papers stacked on either side creating a sandwich and then inserted into the transfer electrode assembly within the tank or semi-dry transfer unit.

3.3.3.5 Primary and secondary antibody incubation for protein detection

3.3.3.5.1 Standard protocol for proteins with a MW > 100kDa

Following transfer of proteins onto PVDF membrane, membrane was incubated in 25ml of blocking solution of 5% non-fat dry milk in 1X Tris-buffered saline (TBS) and 0.1% Tween-20, a polysorbate surfactant or detergent, for 1 hour at room temperature on a flat rocker at a slow steady speed. This step is to prevent non-specific binding and interactions between the membrane and the antibody used for target protein detection. Following blocking stage, blots were washed three times for 5 minutes each with 15 ml of wash buffer consisting of 1X TBS and 0.1% Tween-20 (TBS/T). Blots were then incubated with primary antibody at a dilution of 1:1000 in 10 ml of primary antibody dilution buffer consisting of 1X TBS, 0.1% Tween-20 and 5% BSA at room temperature for 1 hour and then at 4C overnight under gentle rocking. After overnight incubation, blot was washed three times for 5 minutes each with 15 ml of TBS/T under strong agitation to wash off excess primary antibody. Blots were then incubated with appropriate secondary antibody (either anti-rabbit or anti-mouse) at a dilution of 1:10,000 in 10 ml of secondary antibody dilution buffer consisting of 1X TBS, 0.1% Tween-20 and 5% non-fat dry milk at room temperature for 1 hour under gentle rocking. Following secondary antibody incubation, blots were again washed three times for 5 minutes each

with 15 ml of TBS/T under strong agitation to wash off excess secondary antibody to prepare blot for image detection.

Antibodies used in this method are total and phosphorylated mTOR (Ser2448; MW 289 kDa), total and phosphorylated IRS-1 (Ser312; MW 180 kDa) (See Table 3 for detailed antibody information).

3.3.3.5.2 SNAP-id Protocol for proteins with a MW < 100 kDa

Immunodetection system was assembled according to manufacturer's instructions with Millipore SNAP-id-specific blot holders, blot spacers, antibody collection tray, base and vacuum hose together with laboratory-grade vacuum source and Erlenmeyer vaccum flask and plug. Blots were incubated in 15 ml of 1% BSA with 0.1% Tween-20 blocking solution for approximately 30 seconds or until solution dissolved through membrane while under vacuum suction. Antibody collection tray was installed to collect residual primary antibody solution for future use. Blots were incubated in primary antibody at a dilution of 1: 300 in 2 ml of primary antibody dilution buffer consisting of 1% BSA with 0.1% Tween-20 for 10 minutes. Following primary antibody incubation, residual primary antibody was collected while under vacuum suction and then blot was washed three times with 15 ml of wash buffer consisting of 1X TBS with 0.1% Tween-20 while under vacuum suction. Upon full draining of wash buffer from membrane, blots were then incubated with secondary antibody, either anti-rabbit or anti-mouse IgG horseradish peroxidase (HRP) conjugate at a dilution of 1: 3,333 in 2ml of secondary antibody dilution buffer consisting of 0.5% non-fat dry milk with 0.1% Tween-20 for 10 minutes.

Following incubation, blots were washed three times with 15 ml of TBS/T wash buffer under vacuum suction and stored in 1X TBS until ready for image detection. Antibodies used in this method are the following insulin signaling proteins; total and phosphorylated Akt (Ser473; 60 kDa), total and phosphorylated p70S6K (Thr389; 70 kDa), total and phosphorylated eIF4E (Ser209; 25 kDa), total and phosphorylated 4E-BP1 (Ser65; 15, 20 kDa) and lipoprotein lipase antibody (LPL; 53 kDa), CD36 antibody (53 kDa) and loading control beta-actin (42 kDa) (See Table 3 for detailed antibody information).

3.3.3.6 Immunoblot image detection and quantification

Protein detection was facilitated by incubating blot in 1.5 ml of chemiluminescent peroxidase substrate consisting of a 1:2 reagent to buffer solution (Sigma Aldrich, St. Louis, MI, USA) for 2 minutes. Following incubation, blot was exposed to chemiluminescent filtered light for 30 seconds for signal development using an Alpha Innotech FluorChem 5500 imaging system (Santa Clara, CA, USA). Exposed image was converted to JPEG format for densiometry analysis using AlphaEaseFC imaging software, Version 4.0. Blot densiometry analysis was performed using ImageJ software, Version 1.42q (National Institutes of Health, USA). Phosphorylated protein and total protein were normalized and phosphorylated protein was expressed relative to total protein, whereas non-phosphorylated proteins were expressed relative to beta-actin as the loading control.

3.3.3.7 Stripping protocol

Following image detection of phosphorylated protein blots, membranes were stripped and reprobed for detection of total protein in order to determine phosphorylation ratio (phosphorylated protein/total protein) and levels of phosphorylation. Membranes were incubated for 10 minutes in fresh mild stripping buffer, consisting of 1% Tween-20, glycine and SDS with a pH of 2.2, while under strong agitation to strip the previous antibodies from the membrane. Following incubation, the membrane was washed twice with PBS1X for 10 minutes and then washed twice with TBS/T for 5 minutes, both while under strong agitation. Following washing stage, membrane was then blocked and reprobed by incubation with primary antibodies against appropriate total proteins (mTOR, IRS1, S6K, 4EBP1, and eIF4E) following method as previously discussed.

3.3.3.8 Antibodies

Antibody source and usage summary is outlined in Table 3. All insulin signaling phosphorylated and total primary antibodies used were purchased from Cell Signaling (Beverly, MA, USA) including total and phosphorylated mTOR (Ser2448), total and phosphorylated IRS-1 (Ser312), total and phosphorylated Akt (Ser473), total and phosphorylated p70S6K (Thr389), total and phosphorylated eIF4E (Ser209), and total and phosphorylated 4E-BP1 (Ser65). Lpl, GLUT4, CD36 and loading control beta-actin were purchased from Abcam (Cambridge, MA, USA). Anti-rabbit and anti-mouse IgG horseradish peroxidase-conjugated secondary antibodies were purchased from Promega (Madison, WI, USA).

Table 3. Antibody source and usage summary

Primary	Method	1° Dilution	Secondary	2° Dilution	Molecular Weight (kDa)	Source	Catalogue No.
β-actin (loading control)	SNAP-id	1:300	Mouse	1:3000	42	Abcam	Ab8226
GLUT4	SNAP-id	1:300	Mouse	1:3000	54	Abcam	Ab65267
CD36	SNAP-id	1:300	Rabbit	1:3000	53	Abcam	Ab78054
Lpl[1]	SNAP-id	1:300	Mouse	1:3000	53	Abcam	Ab21356
SCD[2]	Standard	1:1000	Mouse	1:10,000	41.5	Abcam	Ab19862
Akt	SNAP-id	1:300	Rabbit	1:3000	60	Cell Signaling	9272
p-Akt Ser^{473}	SNAP-id	1:300	Rabbit	1:3000	60	Cell Signaling	9271
eIF4E[3]	SNAP-id	1:300	Rabbit	1:3000	25	Cell Signaling	9742
p-eIF4E Ser^{209}	SNAP-id	1:300	Rabbit	1:3000	25	Cell Signaling	9741
4EBP1[4]	SNAP-id	1:300	Rabbit	1:3000	15-20	Cell Signaling	9452
p-4EBP1 Ser^{65}	SNAP-id	1:300	Rabbit	1:3000	15-20	Cell Signaling	9451
IRS1[5]	Standard	1:1000	Rabbit	1:10,000	180	Cell Signaling	2382
p-IRS1 Ser^{307}	Standard	1:1000	Rabbit	1:10,000	180	Cell Signaling	2381
mTOR[6]	Standard	1:1000	Rabbit	1:10,000	289	Cell Signaling	2972
p-mTOR Ser^{2448}	Standard	1:1000	Rabbit	1:10,000	289	Cell Signaling	2971
p70S6K[7]	Standard	1:1000	Rabbit	1:10,000	70, 85	Cell Signaling	9202
p-p70S6K Thr^{389}	Standard	1:1000	Rabbit	1:10,000	70, 85	Cell Signaling	9205

[1] Lipoprotein lipase (Lpl); [2] Stearoyl-coA desaturase 1 (SCD); [3] Eukaryotic initiation; factor 4E (eIF4E); [4] 4E-binding protein 1 (4EBP1); [5] Insulin receptor substrate 1 (IRS1); [6]Mammalian target of rapamycin (mTOR); [7] p70S6 Kinase (p70S6K); Ser^{473}, phosphorylated at residue serine-473; Ser^{209}, phosphorylated at residue serine-209; Ser^{65},

phosphorylated at residue serine-65; Ser^{307}, phosphorylated at residue serine-307; Ser^{2448}, phosphorylated at residue serine-2448; Thr^{389}, phosphorylated at residue threonine-389

3.3.4 Fatty acid composition

3.3.4.1 Preparation of fatty acid methyl esters from lipids with boron fluoride-methanol.

Approximately 0.5g of liver and soleus muscle tissue was extracted and saponified according to the Morrison method, where total liver and muscle fatty acids were converted to their methyl esters using boron trifluoride-methanol: hexane: methanol (7:6:7 v/v/v; 1 ml) (Morrison & Smith, 1964).

3.3.4.2 Tissue fatty acid analysis

Prepared liver and muscle fatty acid methyl ester (FAME) concentrations for palmitic, stearic and oleic acids were determined by gas chromatography (GC) with flame ionization (Agilent 6890N, ON, Canada). FAME were separated using a Supelco 2380 (30m x 0.25mm i.d. x 25μm film thickness) with initial temperature of 50°C for 1 min then to 170°C (rate 40°C/min) then to 220°C (rate 8°C/min). Heptadecanoic acid was used as an internal standard and retention times were confirmed by using appropriate pure standards for each fatty acid (Sigma-Aldrich Canada, ON). Fatty acid concentrations were expressed as μg/ml of plasma. The Δ-9 desaturation index of the plasma fatty acid was calculated as the ratio of the total oleic acid pool to the total stearic acid pool.

3.3.4.3 Fatty acid methyl ester deuterium incorporation.

FAME deuterium incorporation was determined using GC separation on a Supelco 2380 column (30m x 0.25mm i.d. x 25μm film thickness) followed by thermal

conversion (pyrolysis) to hydrogen gas at 1450°C online and subsequent isotope ratio of deuterium:hydrogen measured via isotope ratio mass spectrometry (Delta V Plus, Thermo Electron Corporation, Massachusetts, USA). Deuterium enrichment was expressed as delta per mil (δ^2H ‰) versus VSMOW.

3.3.4.4 Fatty acid statistical analysis

All parameters were analyzed by repeated measures ANOVA and Bonferroni post hoc analysis using SPSS (version 11, SPSS, Inc). Linear regression equations were applied to total deuterium incorporation into plasma FAME while best fit polynomial regression equations were applied to FAME deuterium enrichment normalized to pool size. All values are expressed as means ± SEM.

3.4 Statistical analysis

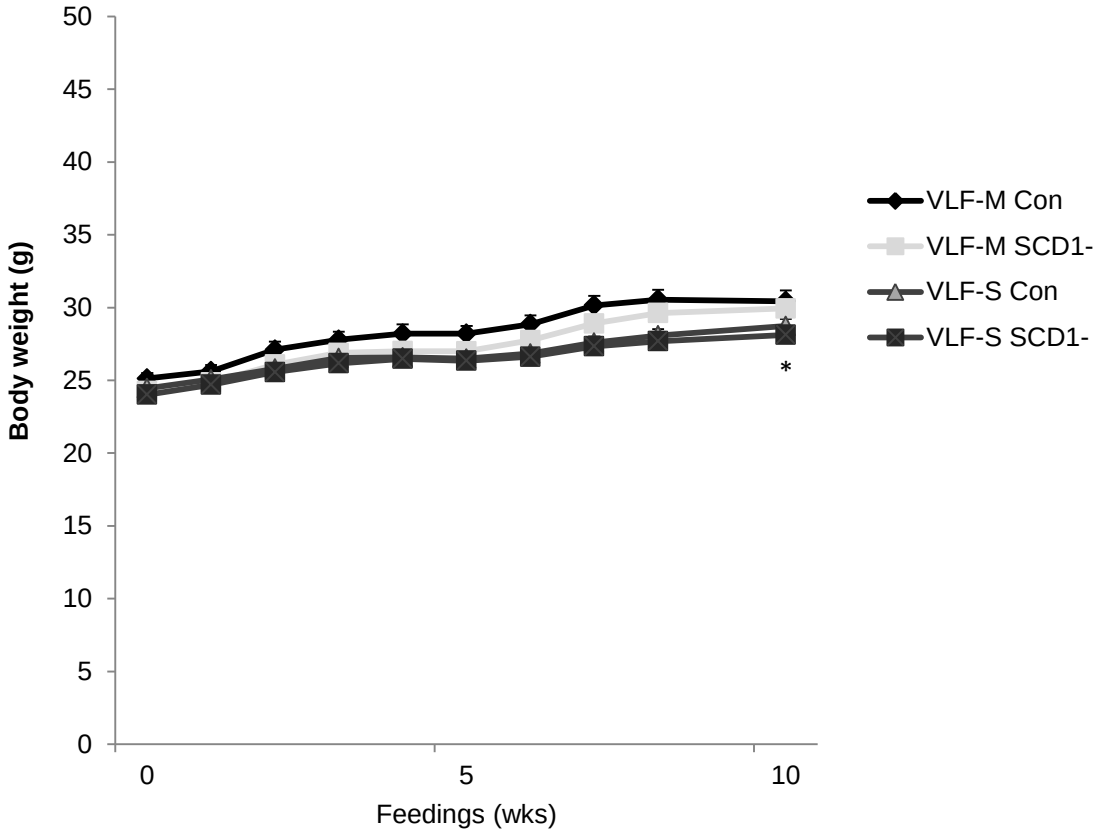
All data was statistically analyzed using SAS software, Version 9.2 (Cary, NC, USA). Comparison of dietary treatments and comparisons of inhibitor treatments were performed using Student's non-parametric t-test. Significance was assessed at $p < 0.05$ and indicated with an asterisk (*). All data are reported as mean values ± SEM.

Chapter 4: RESULTS

4.1 Body weight and food intake

Body weight for all animals increased over the 10 week feeding following the 2 week acclimatization period, however, average weight gain was significantly less ($p=0.02$) in the VLF-S mice compared to the VLF-M mice in the non-fasted state, whereas no significant change in body weights were observed in the fasted state across dietary treatments and no significant changes were observed in the nonfasted state and fasted states when compared within inhibitory treatments (Figure 2a & 2b). Food intake was equal amongst treatments over the 10 week feeding period for both non-fasted and fasted trials (Figure 3a & 3b).

a) Non-fasted group



b) Fasted group

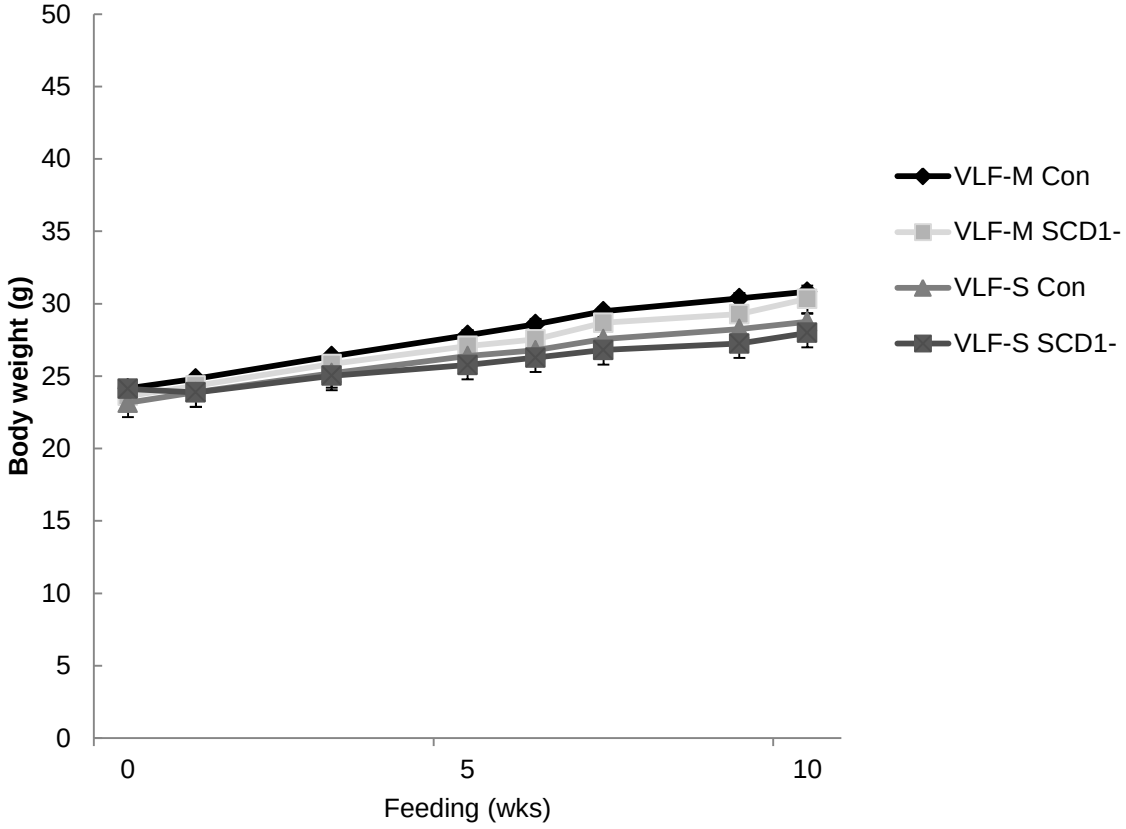
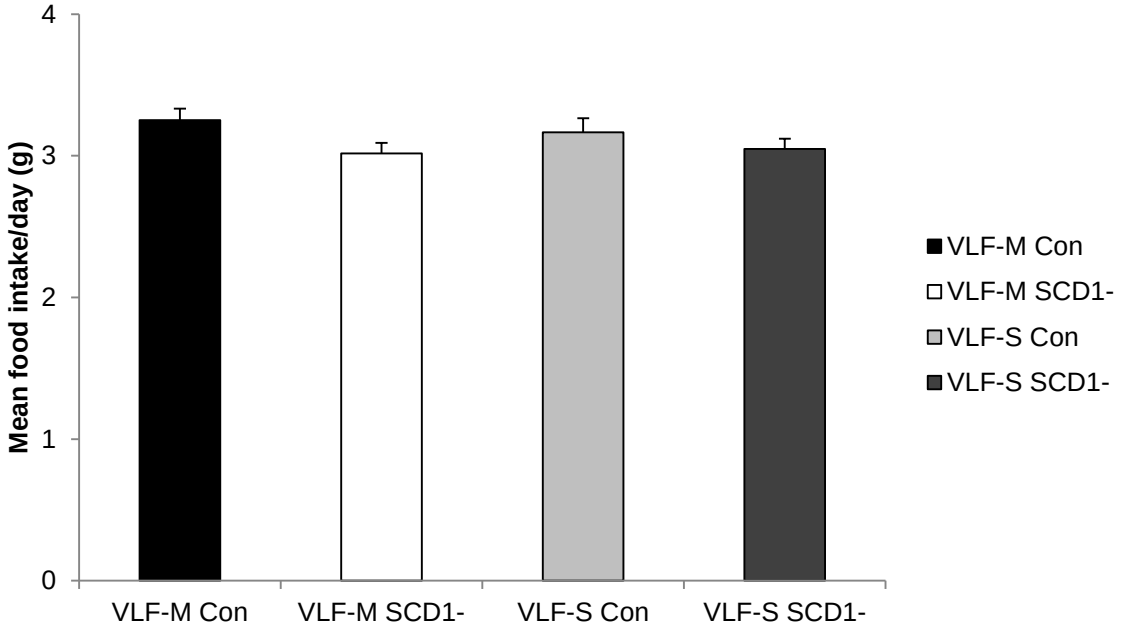


Figure 2. Body weight gain over 10 week study timeline.
Non-fasted group (a); Fasted group (b) body weight gain data are expressed as mean ± SEM (n=8/group).

a) Non-fasted group



b) Fasted group

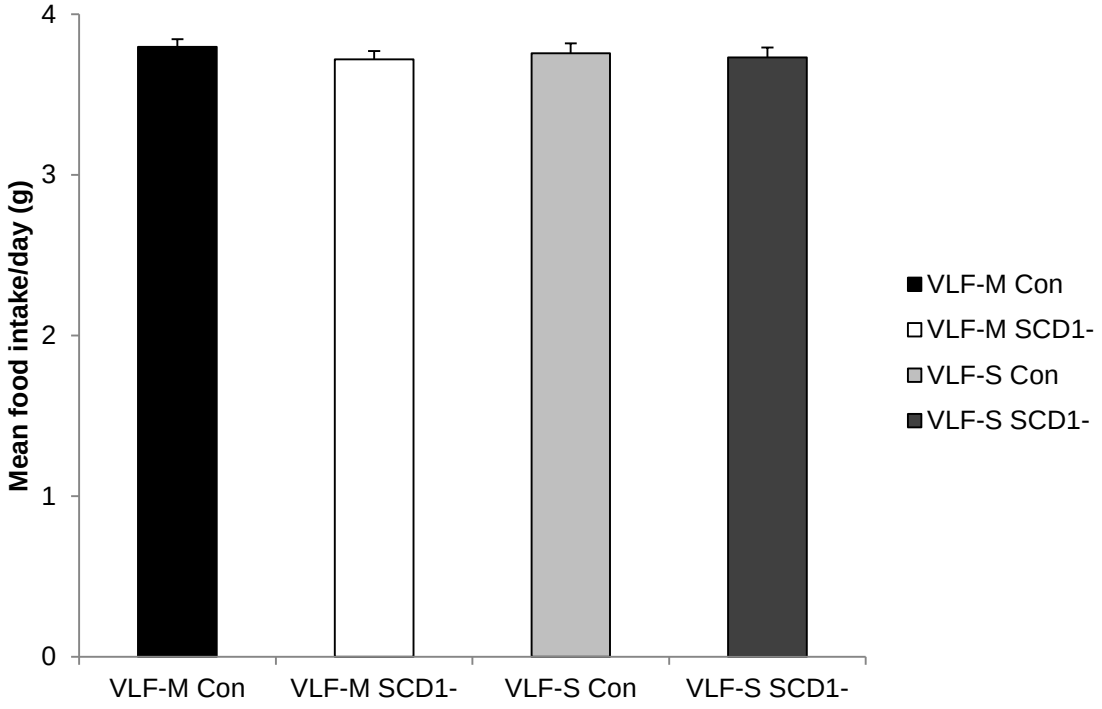
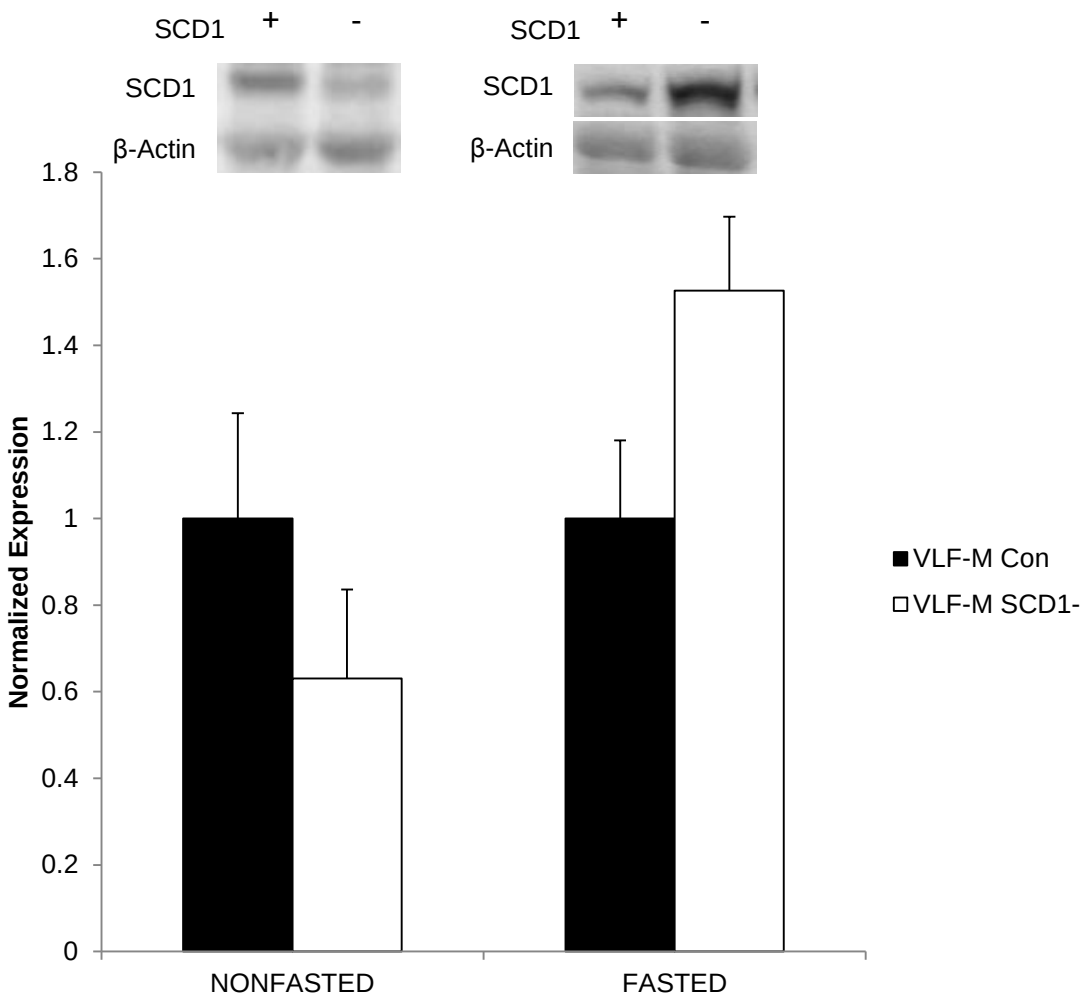


Figure 3. Mean food intake per day.
Non-fasted group (a); Fasted group (b). Values are expressed as mean ± SEM (n=8). Statistical differences among means are indicated as *, assessed at $p < 0.05$.

4.2 Effect of acute SCD1 inhibition on SCD1 activity and *de novo lipogenesis* (DNL)

To confirm whether administration of a small molecule SCD1 inhibitor was effective at down-regulating SCD1 protein activity in peripheral tissues, skeletal muscle SCD1 protein levels were studied and no change was observed in VLF-M mice (Figure 4a.) whereas protein levels were significantly attenuated ($p=0.0007$) in both non-fasted and fasted VLF-S mice (Figure 4b.). As SCD1 is the rate limiting enzyme for DNL and the production of endogenous oleate, the hepatic desaturation index (18:1/18:0) was examined to assess the tissue distribution of oleate and thus the functionality of SCD1 to produce oleate from stearate and was shown to be significantly reduced ($p=0.01$) in all inhibitor groups compared to control mice with normal SCD1 function and is depicted in Figure 5.

a) VLF-M group


SCD1 + -
SCD1
β-Actin
SCD1 + -
SCD1
β-Actin
Normalized Expression
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
NONFASTED
FASTED
VLF-M Con
VLF-M SCD1-

b) VLF-S group

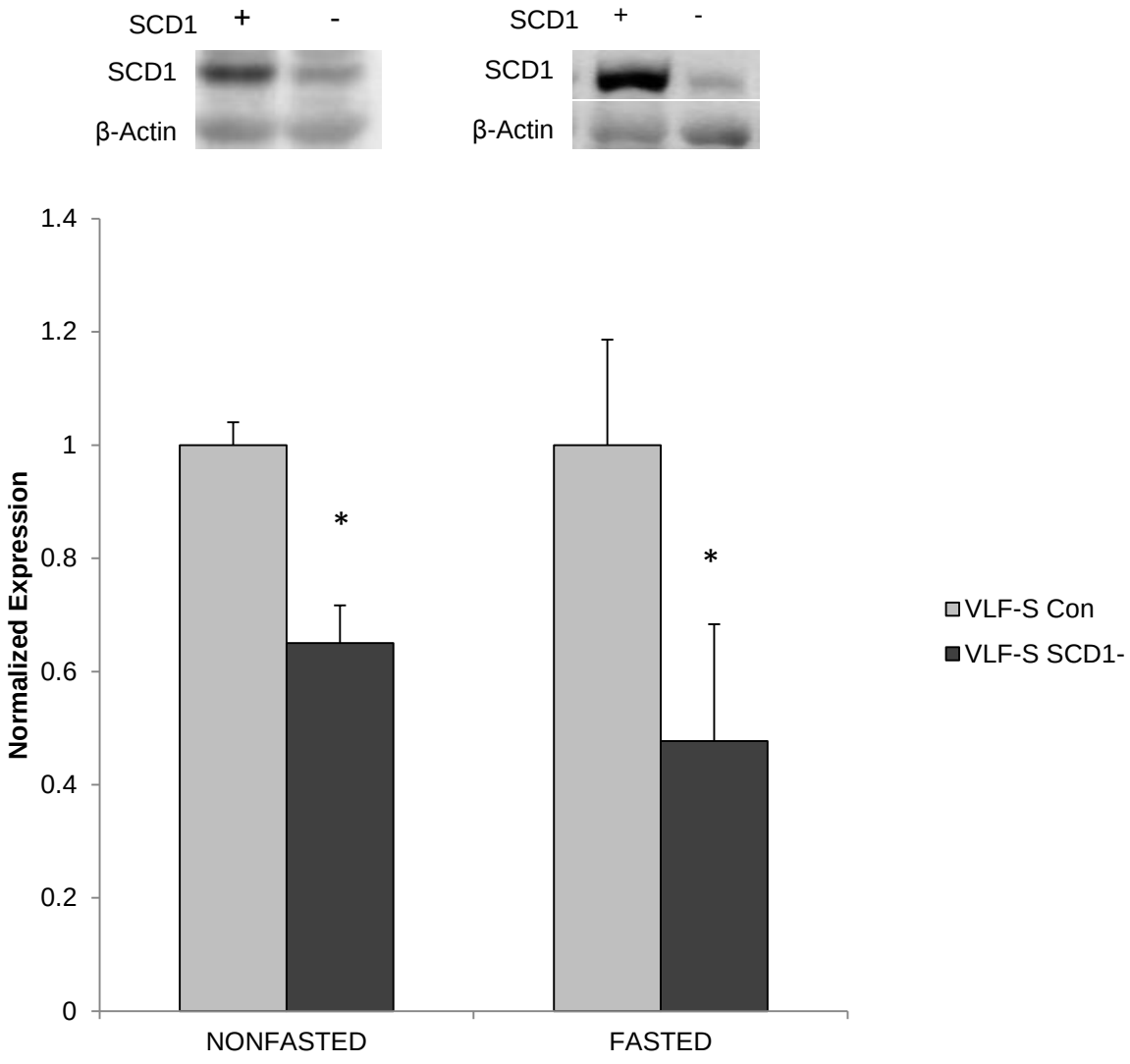


Figure 4. Normalized skeletal muscle protein expression of SCD1 in non-fasted and fasted mice.
VLF-M group (a), VLF-S group (b) normalized SCD1 protein expression are expressed as a mean ratio (arbitrary units) of expressed SCD1 protein over β-actin ± SEM (n=6/group). Statistical differences are indicated as *, assessed at $p < 0.05$. Representative blots are displayed above respective columns.

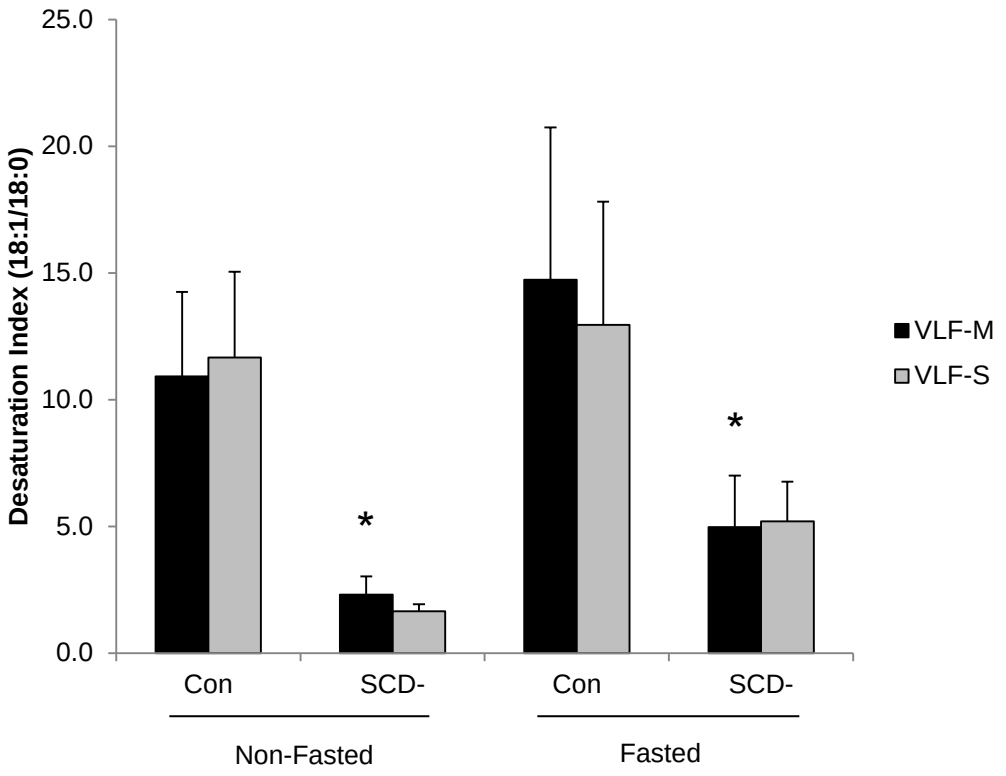


Figure 5. ***De novo lipogenesis*** **(DNL) activity - Hepatic desaturation index.** Values are expressed as mean ratio of hepatic 18:1 over 18:0 ± SEM (n=8/group) to depict DNL activity and SCD1 function. Comparisons are made between Con against SCD- within each dietary treatment. Statistical differences indicated as *, assessed at $p < 0.05$.

4.3 Body composition

Baseline and endpoint (measured at 9 weeks) body composition data are depicted in Figure 6. No significant change in lean body mass (LBM) and fat mass (FM) within dietary treatments were observed at baseline or endpoint, however, significant differences in change in fat mass were seen between diet groups at endpoint ($p=0.0005$). Specifically, fat mass was significantly increased in non-fasted VLF-M-fed mice compared to VLF-S-fed mice (Figure 7a). Although significant changes in fat mass were observed between treatments, significant changes in lean body mass were not observed within or between treatments, however, lean body mass was modified proportionately to the observed change in fat mass (Figure 7b).

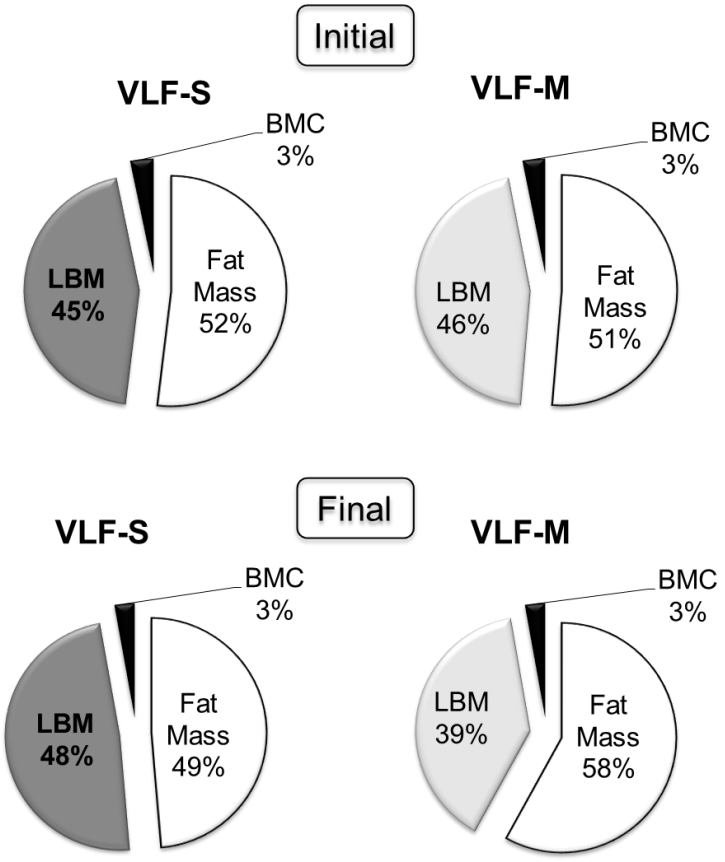
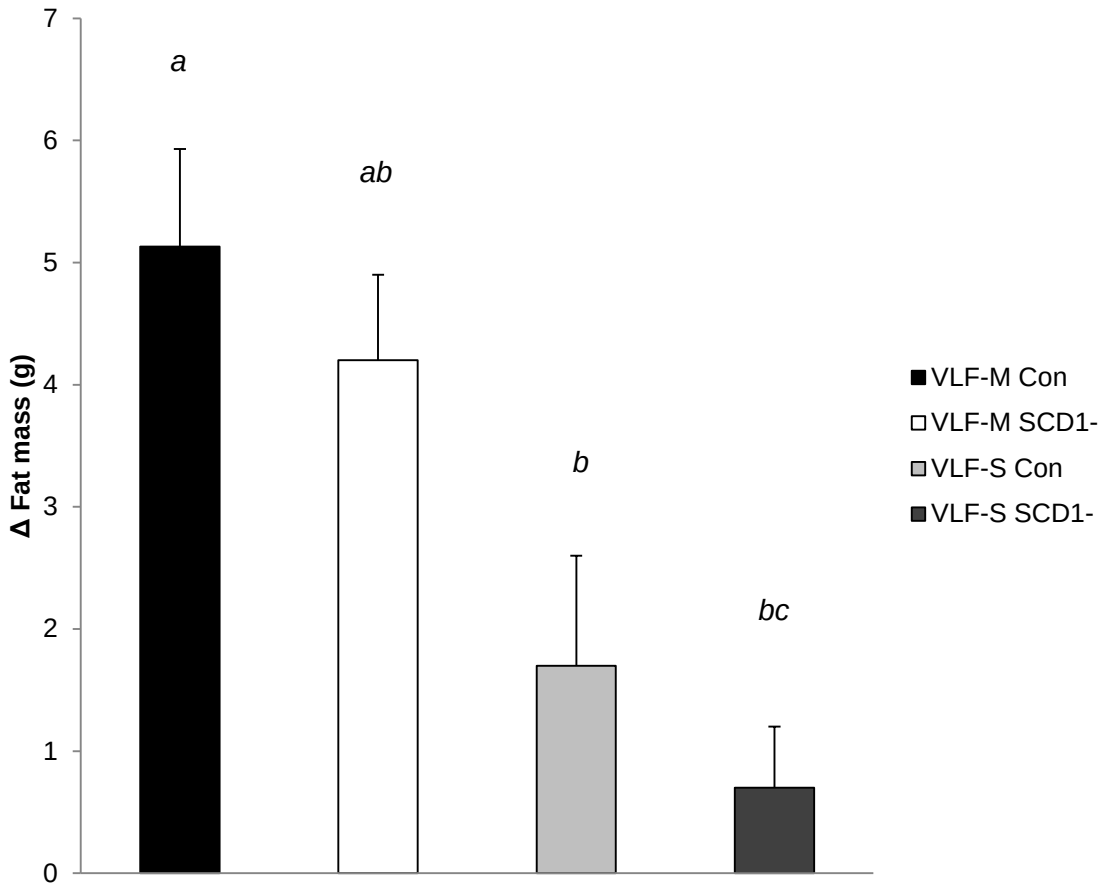


Figure 6. Baseline and endpoint body composition of non-fasted VLF-M and VLF-S-fed mice[1]

[1] bone mineral content (BMC); lean body mass (LBM)

a) Change in fat mass



b) Change in lean body mass

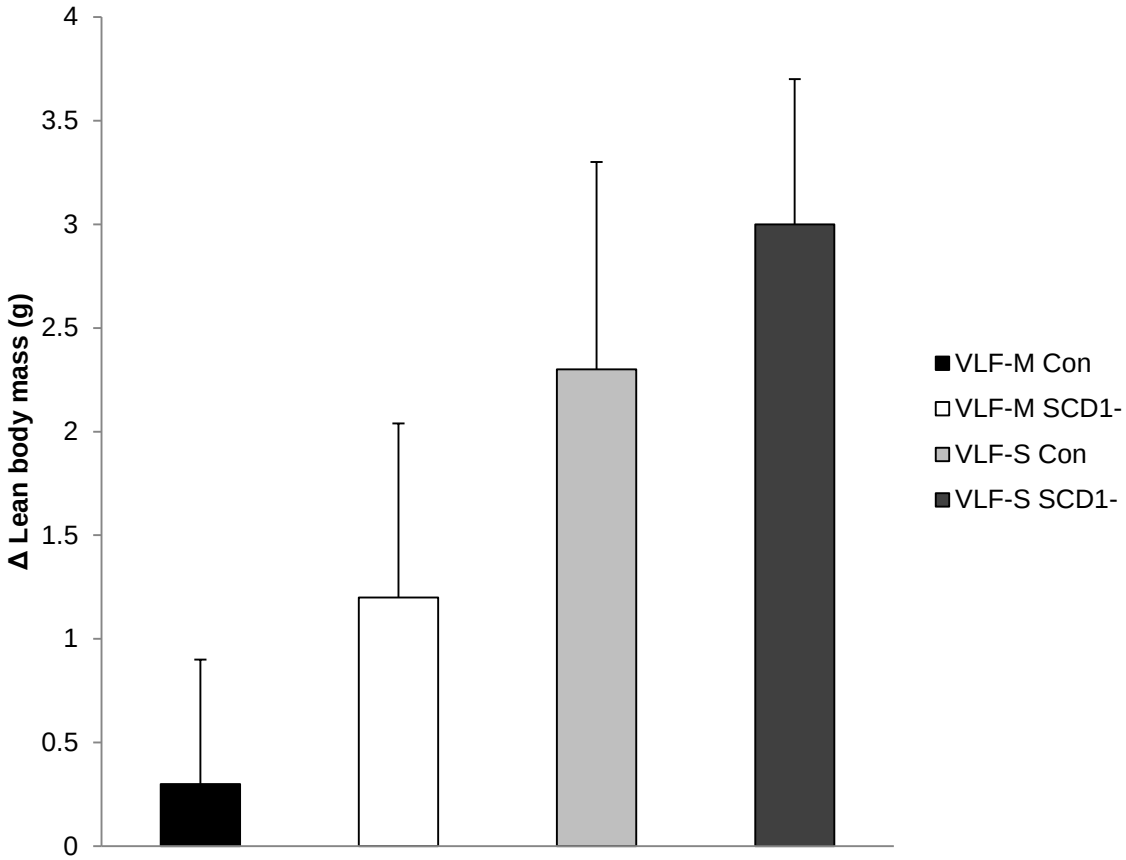


Figure 7. Change in body composition in non-fasted mice with normal SCD1 function in response to VLF-M and VLF-S dietary treatments.
Change in fat mass (a), change in lean body mass (b) values in non-fasted mice in response to dietary treatments. Values are expressed as mean ± SEM (n=8/group). Statistical differences among means are indicated by different letters, assessed at $p < 0.05$.

4.4 Blood biochemistry in response to treatments

4.4.1 Plasma lipid profile

Endpoint values for plasma lipids for each treatment are shown in Table 4. TC levels and non-HDL cholesterol were significantly increased ($p=0.0001$) in inhibitor groups for both dietary treatments and in both energy statuses, non-fasted and fasted states. HDL levels were significantly increased in both non-fasted VLF-M SCD1- ($p=0.01$) and VLF-S SCD1- ($p=0.02$) mice compared to their Con counterparts, as well as fasted VLF-M SCD1- mice ($p=0.02$). No significant differences were observed for plasma TGs between dietary treatments or inhibitor treatments for both energy statuses. Plasma total protein and albumin were shown to significant increase in response to SCD1 inhibition in the non-fasted VLF-M mice ($p=0.0001$), whereas plasma lipase was decreased in both non-fasted and fasted VLF-M mice ($p=0.006$; 0.0001) and no significant differences were observed in non-fasted or fasted VLF-S mice for either parameter.

Table 4. Biochemical parameters in Con and SCD1- mice fed VLF-M and VLF-S diets

			Treatment			Treatment		
Parameter	Units	Energy	VLF-M Con	VLF-M SCD1-	p-value	VLF-S Con	VLF-S SCD1-	p-value
Average body weight	g	NF	27.8 ± 0.48	26.9 ± 0.48	NS	26.4 ± 0.34	26.1 ± 0.33	NS
		F	27.8 ± 0.88	27.1 ± 0.84	NS	26.2 ± 0.71	25.9 ± 0.52	NS
Food intake	g	NF	3.3 ± 0.08	3.0 ± 0.08	NS	3.2 ± 0.1	3.1 ± 0.07	NS
		F	3.8 ± 0.05	3.7 ± 0.05	NS	3.8 ± 0.06	3.7 ± 0.0.06	NS
Plasma								
Total cholesterol	mmol/L	NF	2.9 ± 0.18	4.7 ± 0.18	0.0001	2.3 ± 0.17	4.2 ± 0.36	0.0001
		F	3.2 ± 0.18	5.1 ± 0.28	0.0001	2.7 ± 0.26	3.9 ± 0.40	0.02
Non-HDL cholesterol	mmol/L	NF	0.76 ± 0.56	2.1 ± 0.05	0.0001	0.56 ± 0.07	1.9 ± 0.19	0.0001
		F	0.98 ± 0.08	2.4 ± 0.16	0.0001	0.66 ± 0.08	1.7 ± 0.18	0.0001
HDL cholesterol	mmol/L	NF	2.1 ± 0.13	2.6 ± 0.13	0.01	1.8 ± 0.11	2.3 ± 0.19	0.02
		F	2.2 ± 0.11	2.7 ± 0.14	0.02	2.0 ± 0.18	2.2 ± 0.23	NS
Triglycerides	mmol/L	NF	0.7 ± 0.08	0.7 ± 0.05	NS	0.8 ± 0.13	0.8 ± 0.08	NS
		F	0.7 ± 0.1	0.7 ± 0.02	NS	0.7 ± 0.05	0.6 ± 0.02	NS
Glucose	mmol/L	NF	17 ± 0.72	16 ± 0.81	NS	17 ± 0.50	14 ± 0.86	0.02
		F	15 ± 1.3	13 ± 0.56	NS	13 ± 1.0	11 ± 0.99	NS
Insulin	μg/L	NF	1.2 ± 0.09	1.1 ± 0.06	NS	1.2 ± 0.08	1.3 ± 0.07	NS
		F	1.2 ± 0.36	1.0 ± 0.09	NS	1.0 ± 0.1	0.72 ± 0.009	NS
Total Protein	g/L	NF	49 ± 0.31	52 ± 0.38	0.0001	49 ± 1.0	52 ± 0.78	NS
		F	44 ± 0.78	46 ± 1.2	NS	43 ± 2.1	44 ± 1.1	NS
Albumin	g/L	NF	21 ± 0.26	22 ± 0.18	0.0001	21 ± 0.68	21 ± 0.56	NS
		F	21 ± 0.61	20 ± 0.6	NS	19 ± 1.2	19 ± 0.75	NS
Lipoprotein Lipase	U/L	NF	712 ± 37	573 ± 17	0.006	690 ± 33	676 ± 66	NS
		F	524 ± 16	428 ± 7.4	0.0001	479 ± 30	521 ± 77	NS

Values are expressed as mean ± SEM (n=8/group). NF, non-fasted; F, fasted. Comparisons made between Con and SCD1- group of each dietary treatment. Statistical significance assessed at $p < 0.05$, otherwise noted as not significant (NS).

4.4.2 Plasma glucose and insulin levels

Endpoint glucose and insulin levels are displayed in Table 4 and Figure 8 and Figure 9, respectively. VLF-S SCD1- non-fasted mice showed a significant decrease ($p=0.02$) in plasma glucose, whereas no significant difference was observed in other treatments. No significant difference in insulin levels was shown between dietary treatments or between inhibitor treatments.

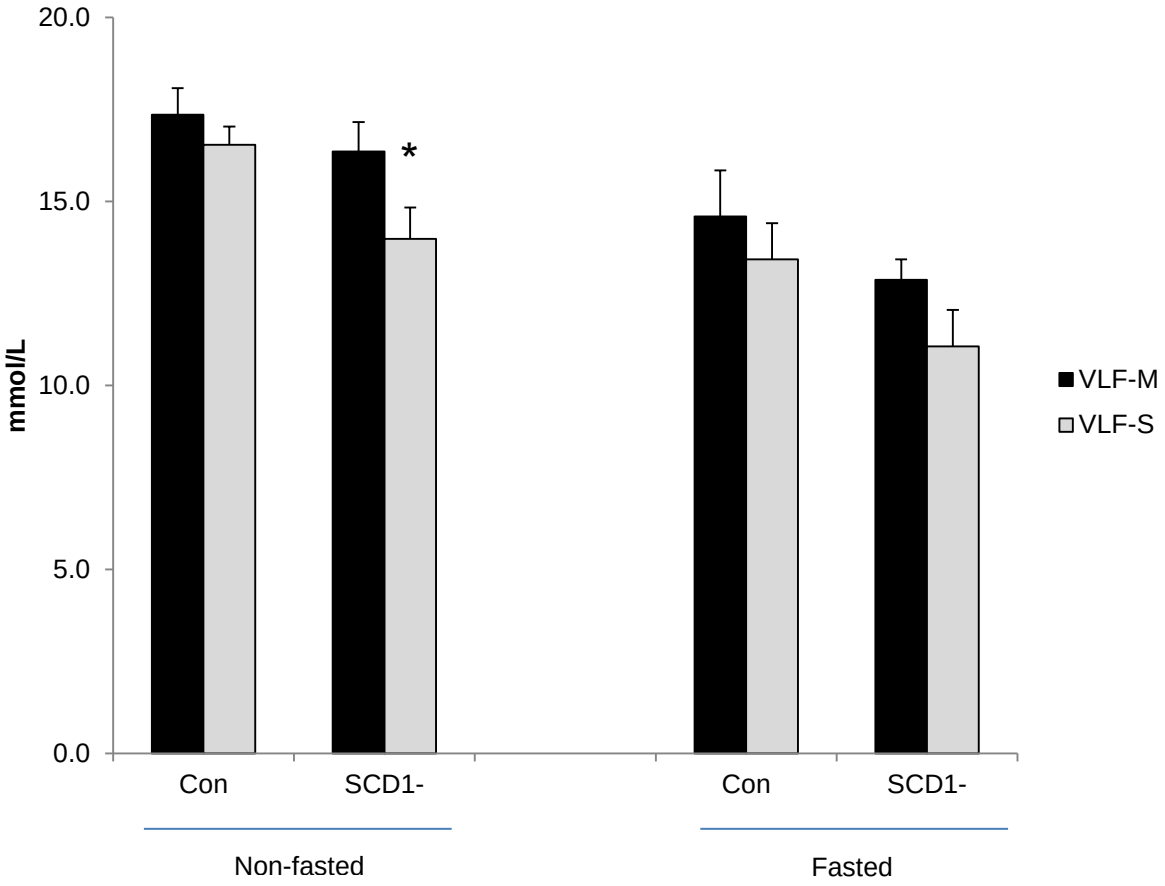


Figure 8. Plasma glucose.
Plasma glucose concentrations are expressed as mean ± SEM (n=8/group). Statistical differences are indicated as *, assessed at $p < 0.05$, compared to SCD1- counterparts.

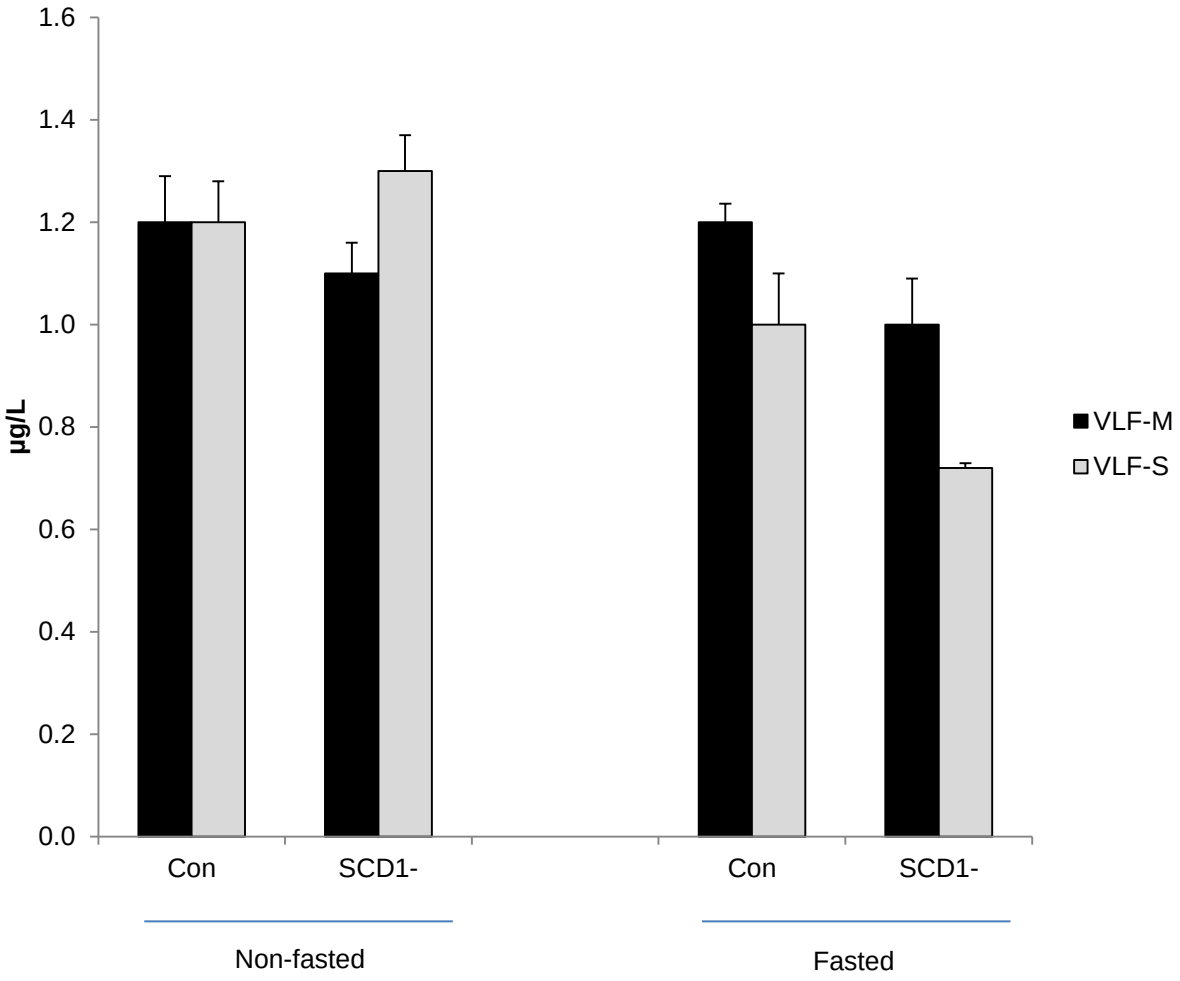


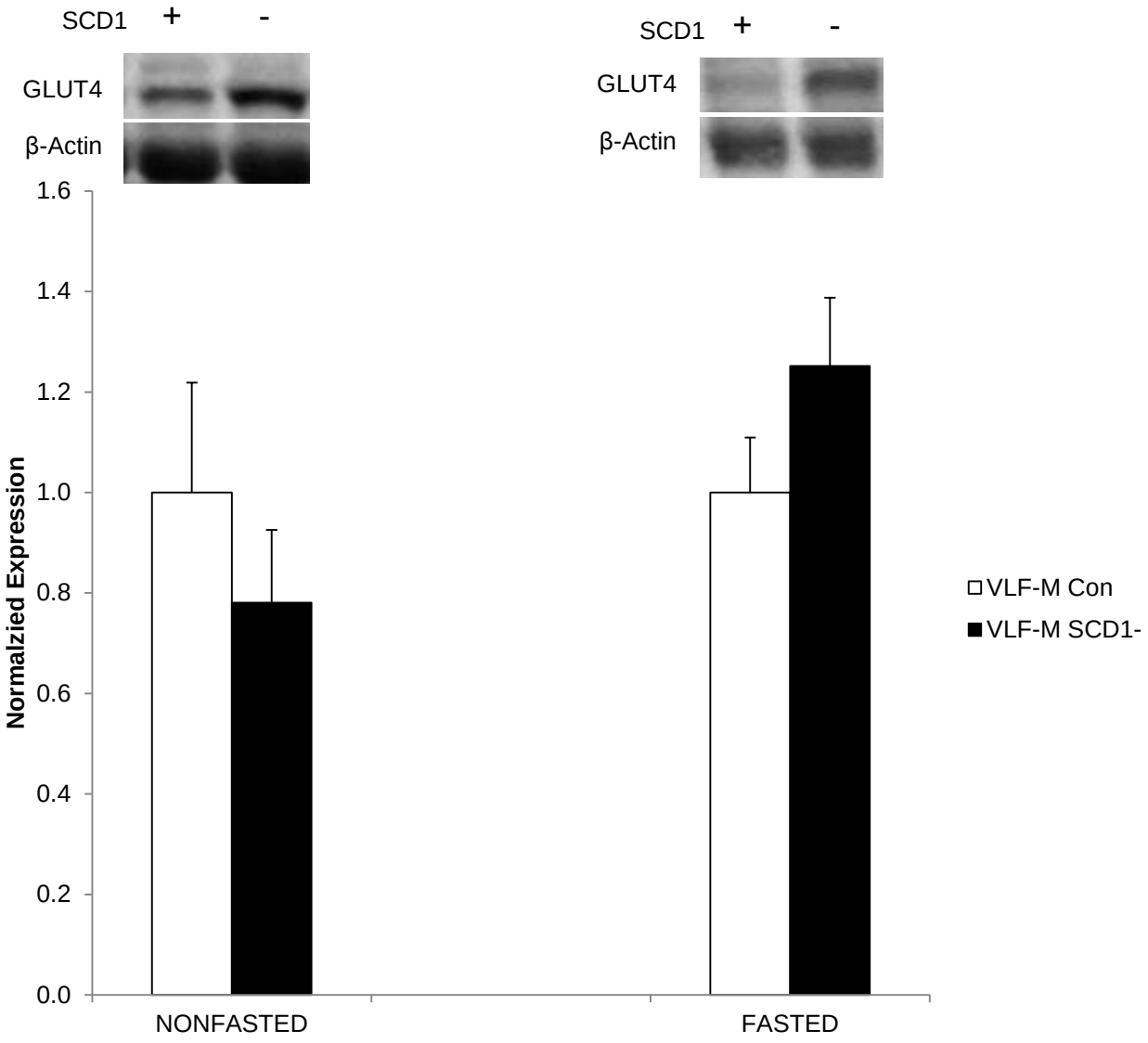
Figure 9. Plasma insulin.
Plasma insulin concentrations are expressed as mean ± SEM (n=8/group). Statistical differences are indicated as *, assessed at $p < 0.05$, compared to SCD1- counterparts.

4.5 Immunoblotting

4.5.1 Glucose uptake

To investigate whether the significant decrease in plasma glucose levels was due to improved insulin signaling and increased glucose uptake, phosphorylated levels of IRS-1 protein and total GLUT4 protein content were measured. Normalized GLUT4 protein expression for VLF-M and VLF-S mice is displayed in Figure 10a and 10b, respectively. Surprisingly, undetectable levels of serine-phosphorylated IRS-1 were observed amongst all treatments compared to total IRS-1, the first major cellular signaling protein that is stimulated by insulin after insulin receptor and initiates the downstream activation of this insulin signaling cascade. This lack of effect on IRS-1 phosphorylation was observed in congruence with a significant decrease ($p=0.05$) in total GLUT4 protein content in both non-fasted and fasted VLF-S SCD1- groups, whereas total GLUT4 protein remained unchanged in VLF-M SCD1- mice.

a) VLF-M group


SCD1 + -
GLUT4
β-Actin
SCD1 + -
GLUT4
β-Actin
Normalzied Expression
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
NONFASTED
FASTED
VLF-M Con
VLF-M SCD1-

b) VLF-S group

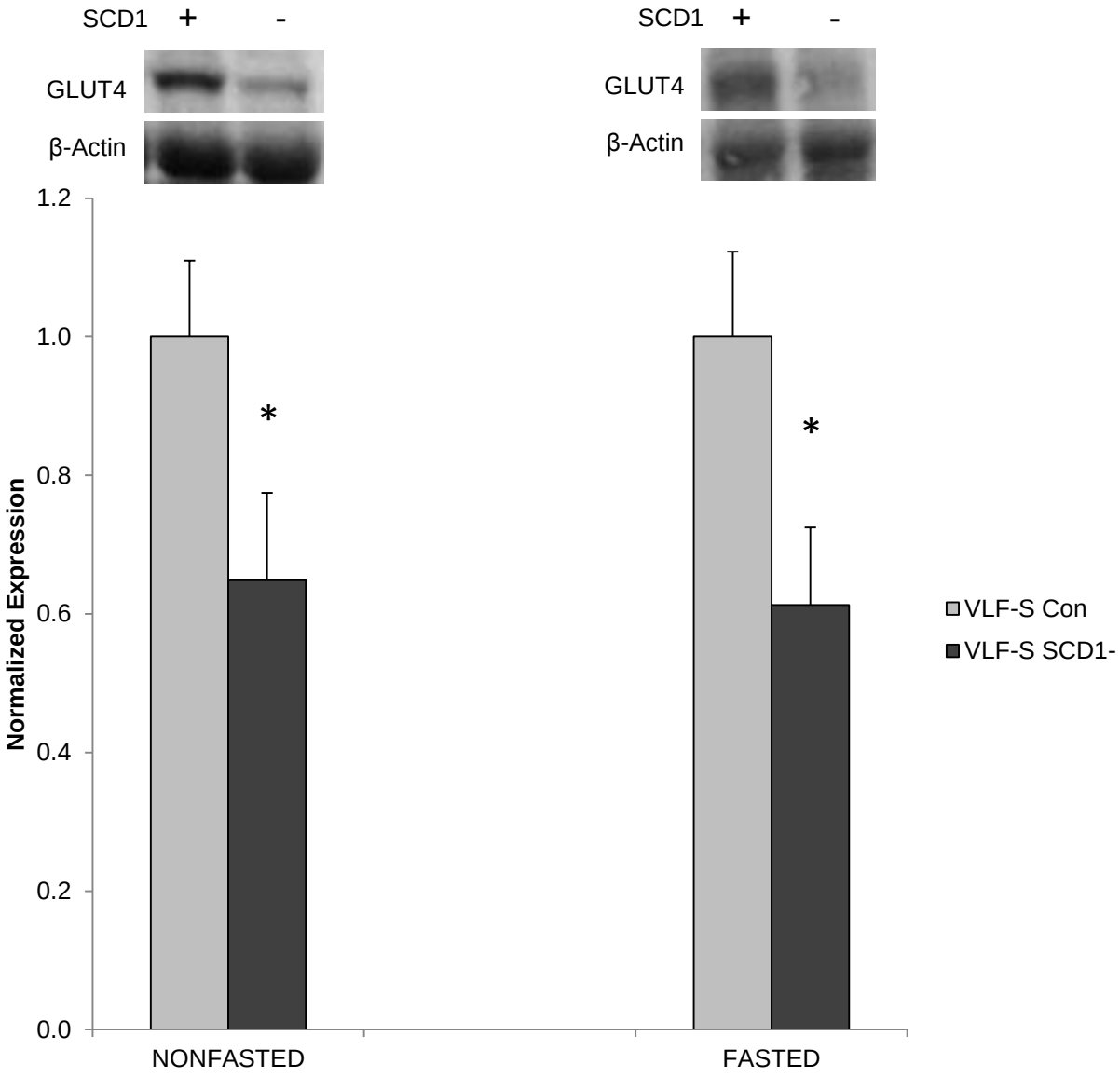
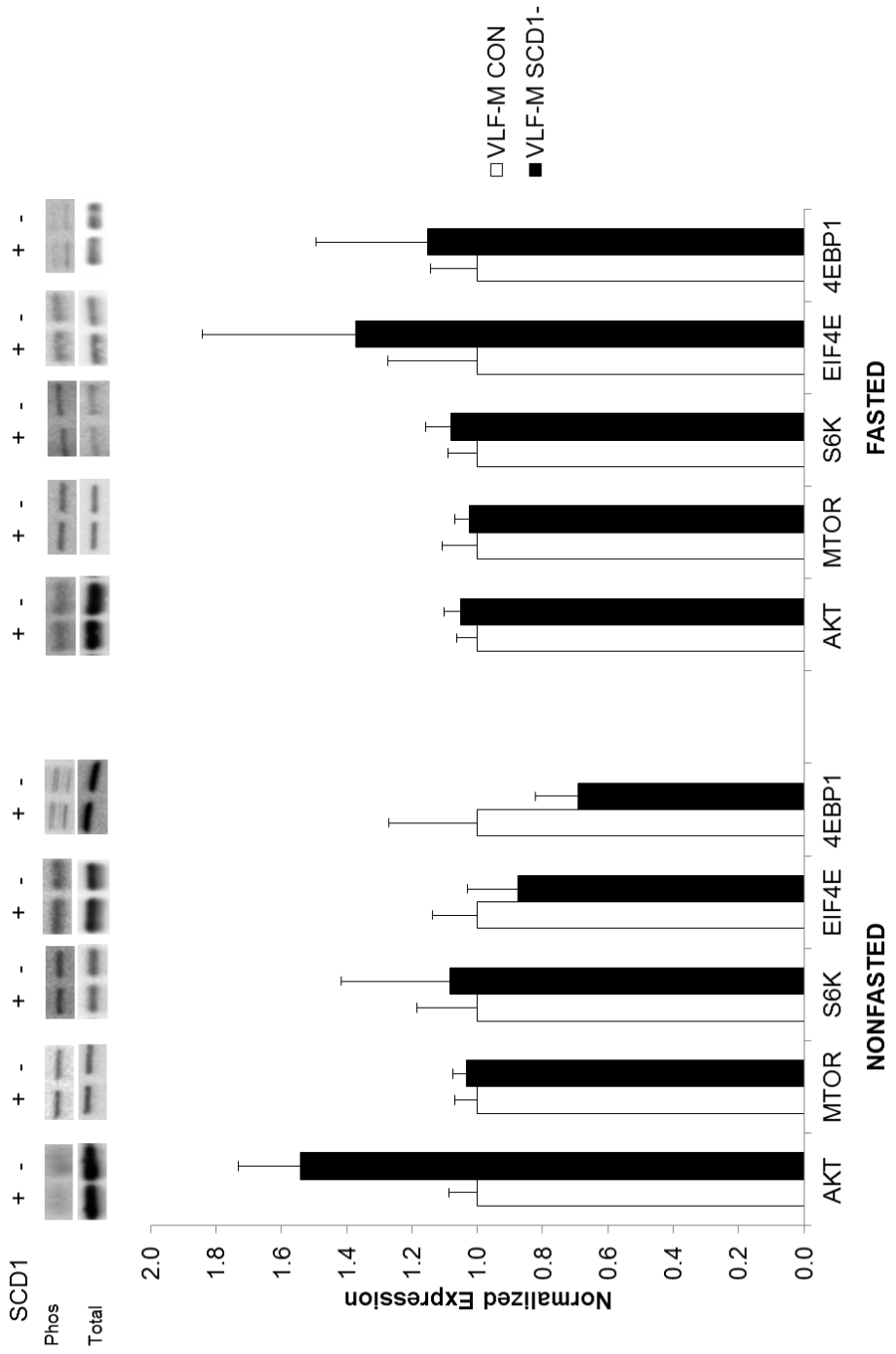


Figure 10. Normalized skeletal muscle protein expression of GLUT4 in non-fasted and fasted mice.
VLF-M group (a), VLF-S group (b) normalized protein expression are expressed as a mean ratio (arbitrary units) of GLUT4 protein over loading control β-actin ± SEM (n=6/group). Statistical differences indicated as *, assessed at $p < 0.05$. Representative blots are displayed above respective columns.

4.5.2 Protein phosphorylation of the Akt/mTOR/S6k pathway

We investigated changes in phosphorylation within the Akt/mTOR/S6K insulin signaling cascade and observed that surprisingly there were no significant changes within major cofactors that lead to mRNA and cap-dependent RNA translation for cell proliferation, specifically, mTOR, S6K, eIF4E and 4EBP1, however, this was not the same observation seen in total and phosphorylated Akt (Figure 11a & b). Phosphorylation of Akt Ser473 showed a strong tendency to increase in response to SCD1 inhibition in the fasted VLF-S group ($p=0.06$) but otherwise was not shown to increase in any other treatment. When non-fasted and fasted treatment groups were compared, as expected, insulin signaling proteins showed significant decreases in phosphorylation in response to fasting.

a) VLF-M group


VLF-M CON
VLF-M SCD1-
Normalized Expression
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
AKT
MTOR
S6K
EIF4E
4EBP1
NONFASTED
AKT
MTOR
S6K
EIF4E
4EBP1
FASTED
SCD1
Phos
Total
+ -

b) VLF-S group

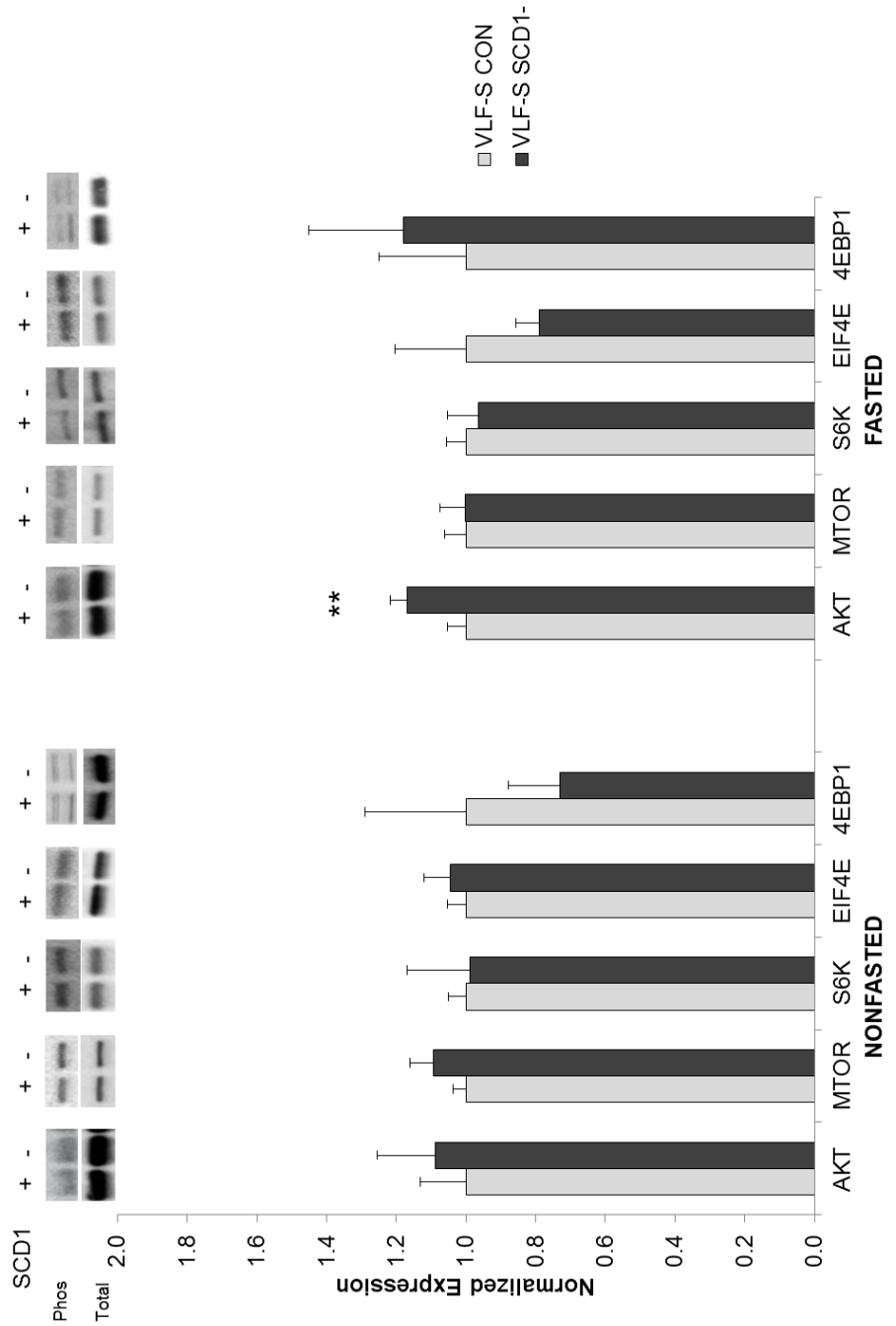


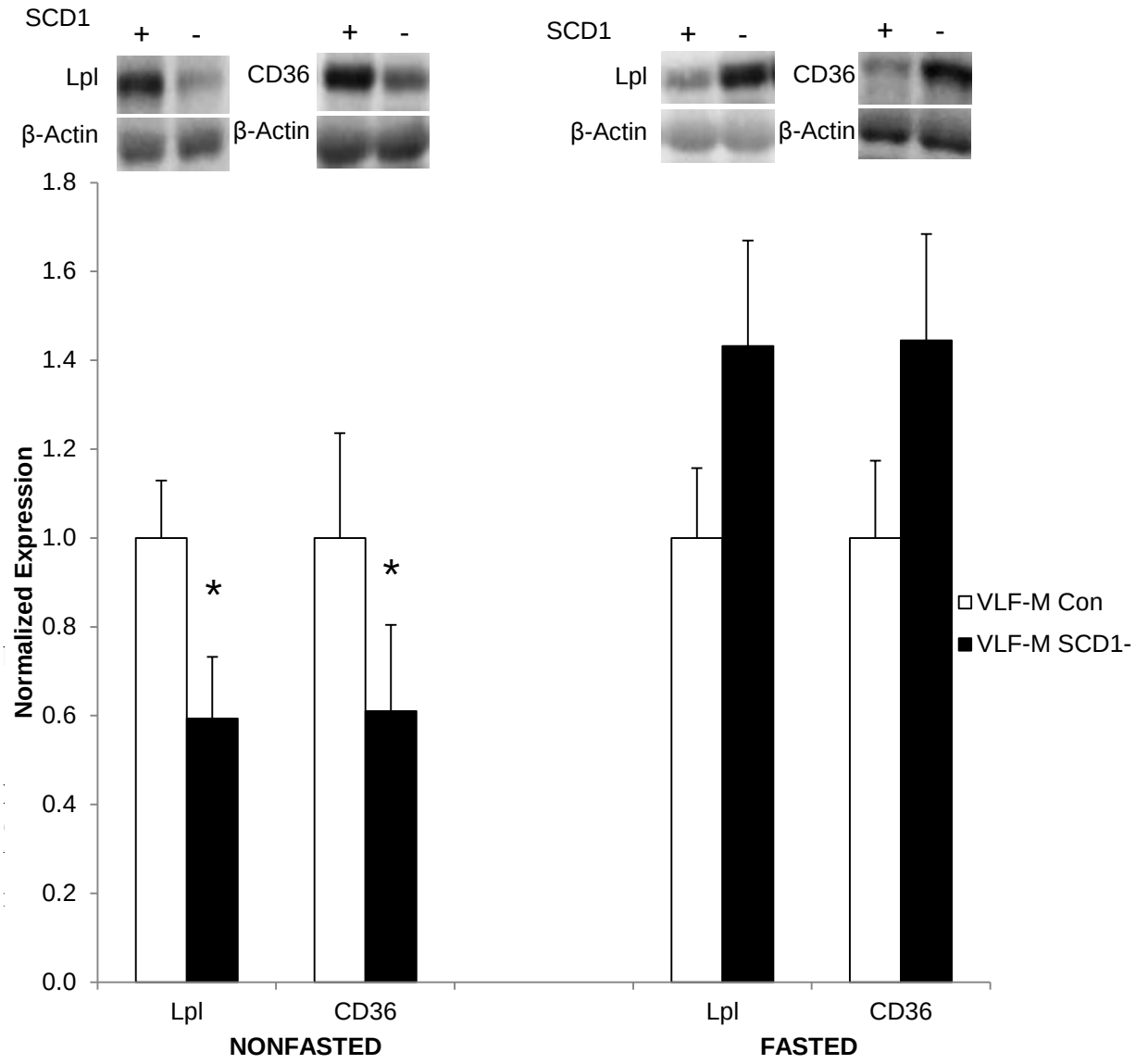
Figure 11. Skeletal muscle phosphorylation of key signaling proteins within the Akt/mTOR/S6K insulin signaling network.
VLF-M group (a), VLF-S group (b) normalized expression of skeletal muscle protein phosphorylation are expressed as a mean ratio (arbitrary units) of phosphorylated protein over total protein ± SEM (n=5-6/group). Statistical differences indicated as **, assessed at $p > 0.05$. Representative blots are displayed above respective columns.

4.5.3 Nutrient handling

Protein expression of key nutrient handling proteins, Lpl and CD36, were measured and are presented in Figure 12. Interestingly, it was shown that expression of Lpl significantly decreased ($p=0.02$) in response to SCD1 inhibition in non-fasted VLF-M mice (Fig. 12a), but were decreased ($p=0.002$) in fasted VLF-S mice (Fig. 12b). Concurrently, the same trend occurred in CD36 expression with total CD36 protein content decreased ($p=0.04$) in non-fasted VLF-M mice and in fasted VLF-S mice ($p=0.009$) in response to SCD1 inhibition.

As it was determined that phosphorylation of cell signaling proteins within the Akt/mTOR/S6K network were not significantly changed across treatments although there was an abundance of FFAs in SCD1- groups, it was hypothesized that the expression of specific proteins that facilitate lipoprotein hydrolysis and transport may be modified in response to acute SCD1 inhibition and altered lipid partitioning.

a) VLF-M group


SCD1
+
-
Lpl
β-Actin
CD36
β-Actin
SCD1
+
-
Lpl
β-Actin
CD36
β-Actin
Normalized Expression
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Lpl
CD36
NONFASTED
Lpl
CD36
FASTED
VLF-M Con
VLF-M SCD1-

b) VLF-S group

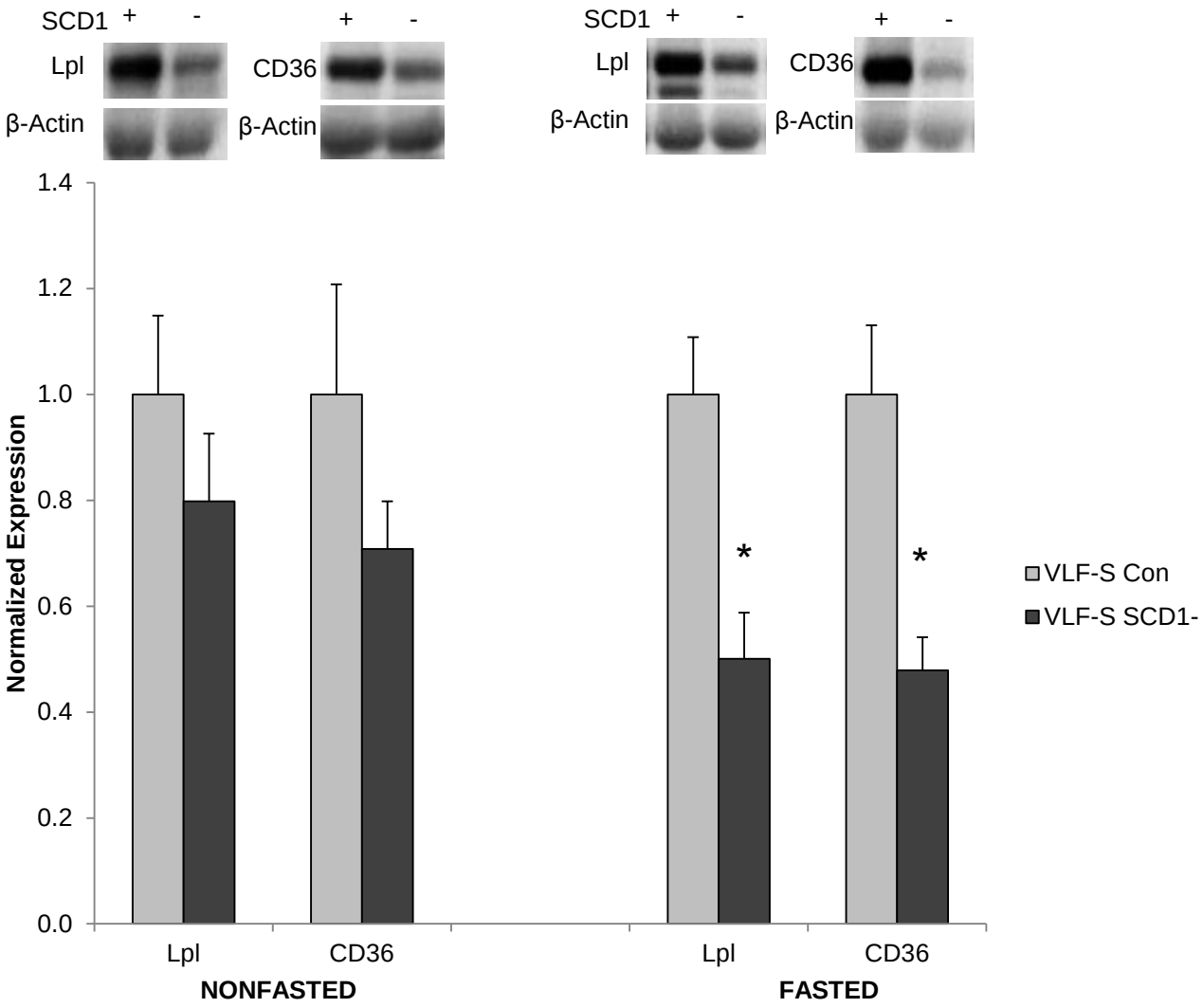


Figure 12. Normalized skeletal muscle protein expression of nutrient handling proteins, Lpl and CD36, of non-fasted and fasted mice.
VLF-M group (a), VLF-S group (b) normalized protein expression are expressed as a mean ratio (arbitrary units) of Lpl and CD36 protein over loading control β-actin ± SEM (n=6/group). Statistical differences indicated as *, assessed at $p < 0.05$. Representative blots are displayed above respective columns.

4.6 Fatty acid composition

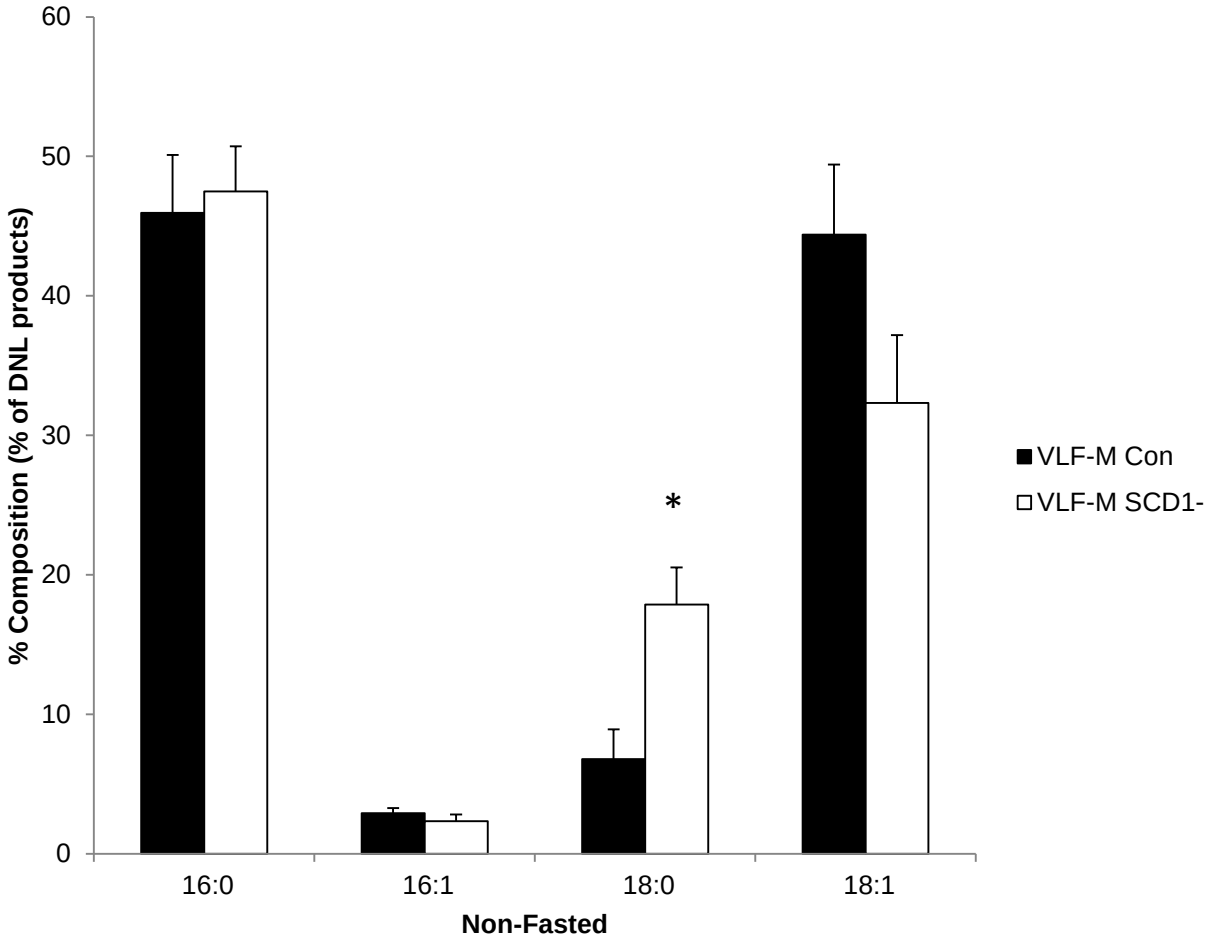
To elucidate whether the metabolic effects observed were associated with a modification in the fatty acid composition of key tissues in response to the saturation of the diets or the perturbation of SCD1 activity, the 16:0, 16:1, 18:0 and 18:1 fatty acid content of liver and soleus muscle tissue in the four treatment groups was examined and is presented in Figure 13 and 14, respectively.

Consistently, with SCD1 inhibition levels of MUFAs, 16:1 and 18:1, are decreased concurrent with increased levels of SFAs, 16:0 and 18:0, in both liver and soleus muscle signifying effective inhibition of SCD1 function in the liver, but also disturbed lipid portioning in peripheral tissues.

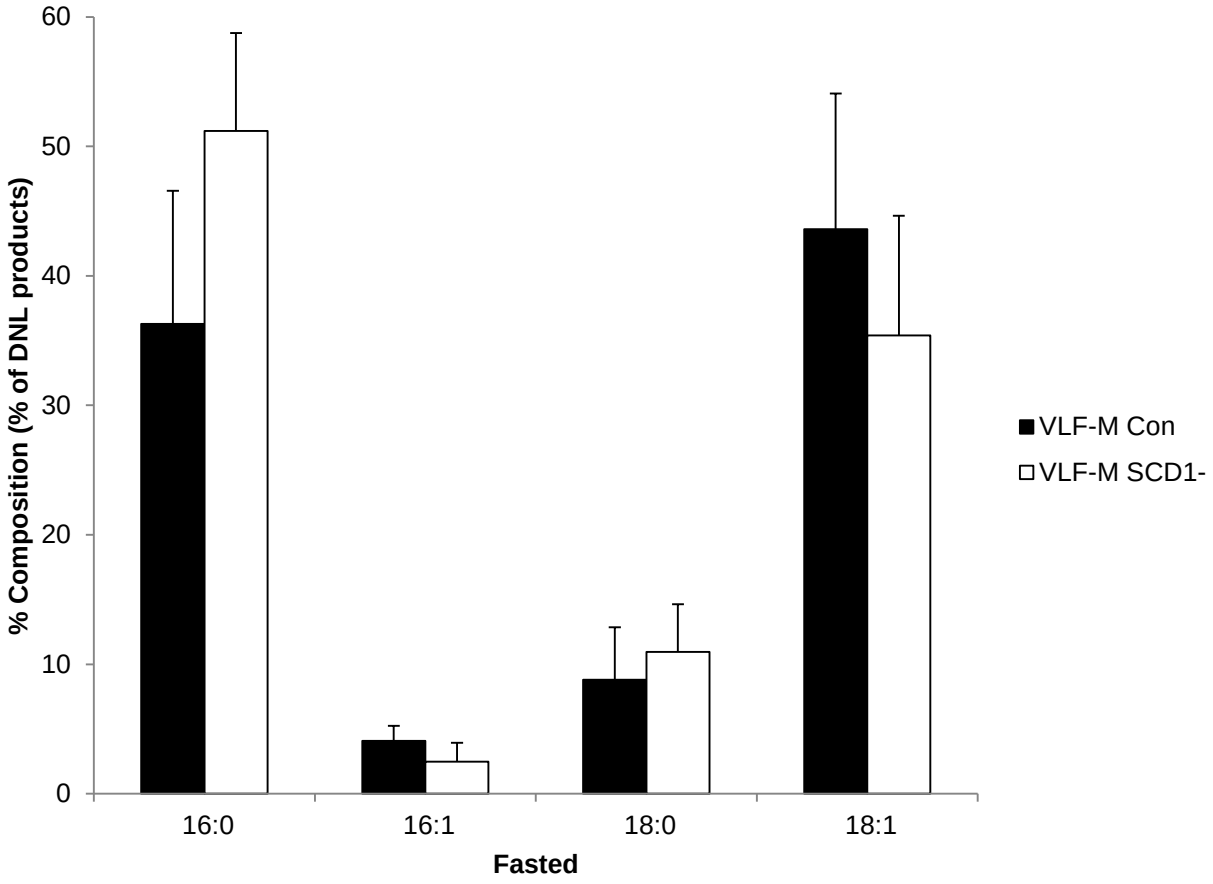
Interestingly, fatty acid composition was significantly modified in liver tissue only in the nonfasted energy state, whereas soleus muscle fatty acids were only significantly different in the fasted state and only within the VLF-S diet. It was observed in liver tissue that levels of 18:0 were significantly increased in SCD1- mice fed both VLF-M ($p=0.009$) and VLF-S ($p=0.0001$) diets, whereas levels of 18:1 were not shown to be significantly different amongst groups. In addition, levels of 16:1 were shown to be significantly decreased ($p=0.001$) in liver tissue in response to SCD1 inhibition in VLF-S mice, although no significant difference was observed for liver levels of 16:0. Alternatively, the compositional levels of fatty acids in soleus muscle were only shown to be significantly modified in the fasted VLF-S group, with levels of 16:0 significantly increased ($p=0.03$) with SCD1 inhibition, however, in congruence with liver tissue, levels of 16:1 also tended to decrease but to a much lesser extent ($p=0.09$) in SCD1- muscle tissue. Lastly, the % of DNL products that were 18:1 were significantly decreased

($p=0.05$) in SCD1- muscle tissue, whereas no significant differences were observed in 18:0 levels in response to SCD1 inhibition.

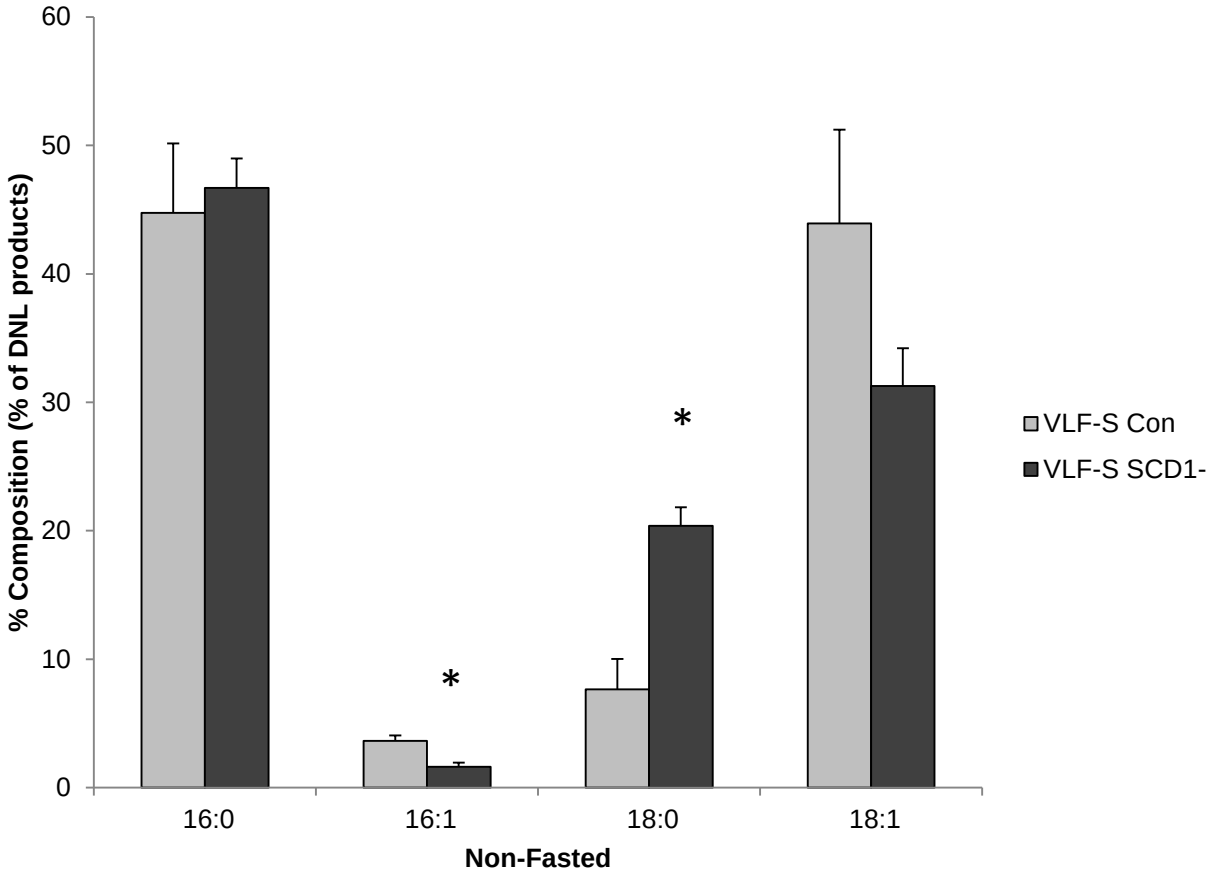
a) Hepatic fatty acid composition – VLF-M Non-fasted group



b) Hepatic fatty acid composition – VLF-M Fasted group



c) Hepatic fatty acid composition – VLF-S Non-fasted group



d) Hepatic fatty acid composition – VLF-S Fasted group

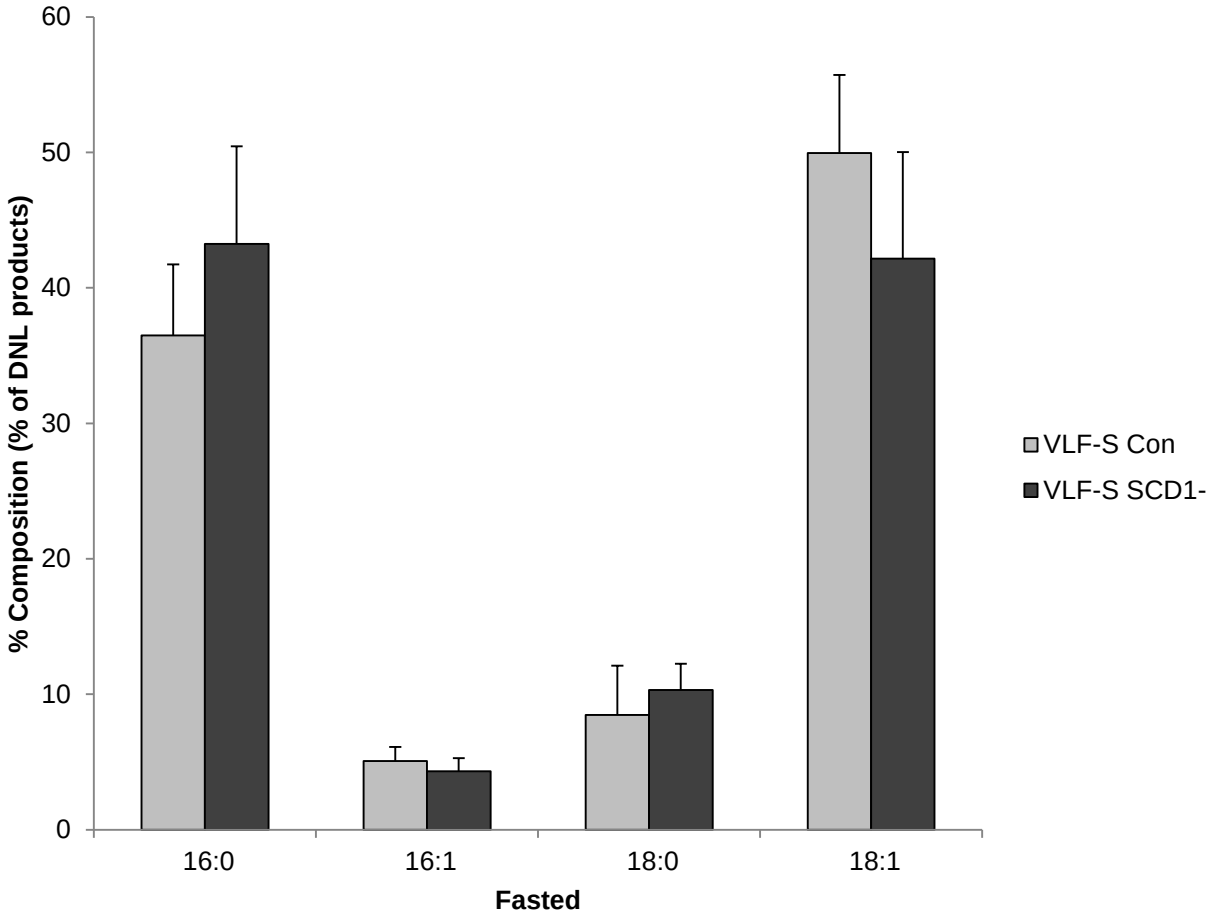
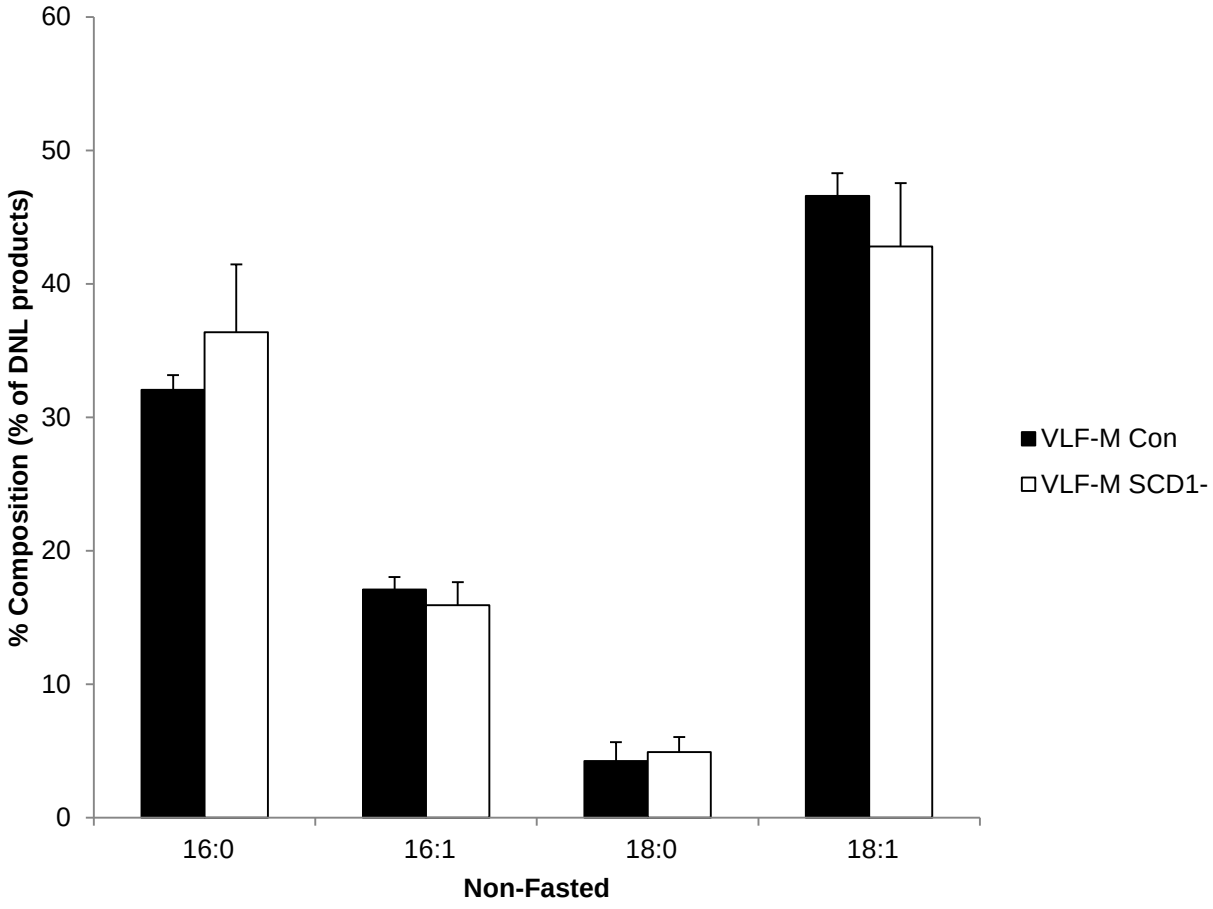
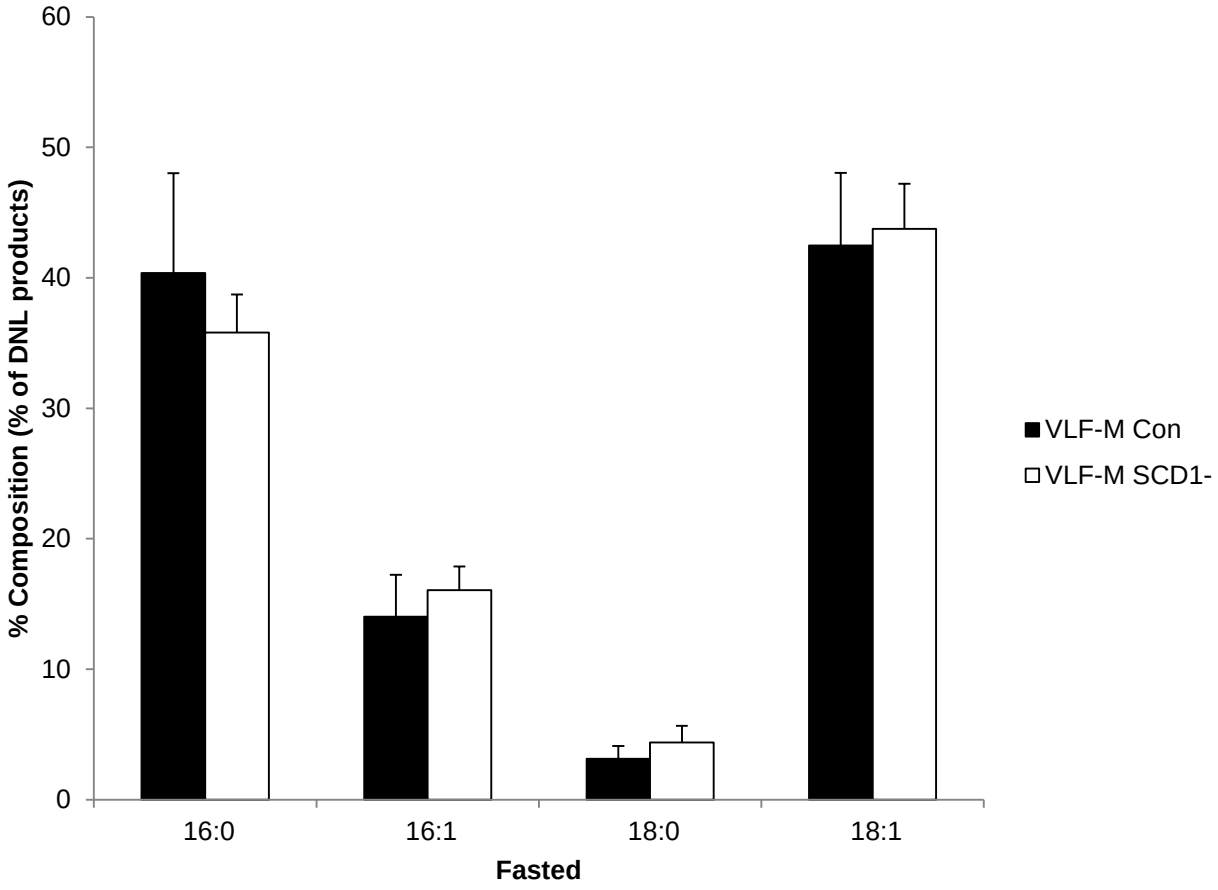


Figure 13. Hepatic TG – % of DNL products.
VLF-M Non-fasted (a), VLF-M Fasted (b), VLF-S Non-fasted (c), VLF-S Fasted (d) Values are expressed as mean ± SEM (n=8/group). Statistical differences indicated as *, assessed at $p < 0.05$.

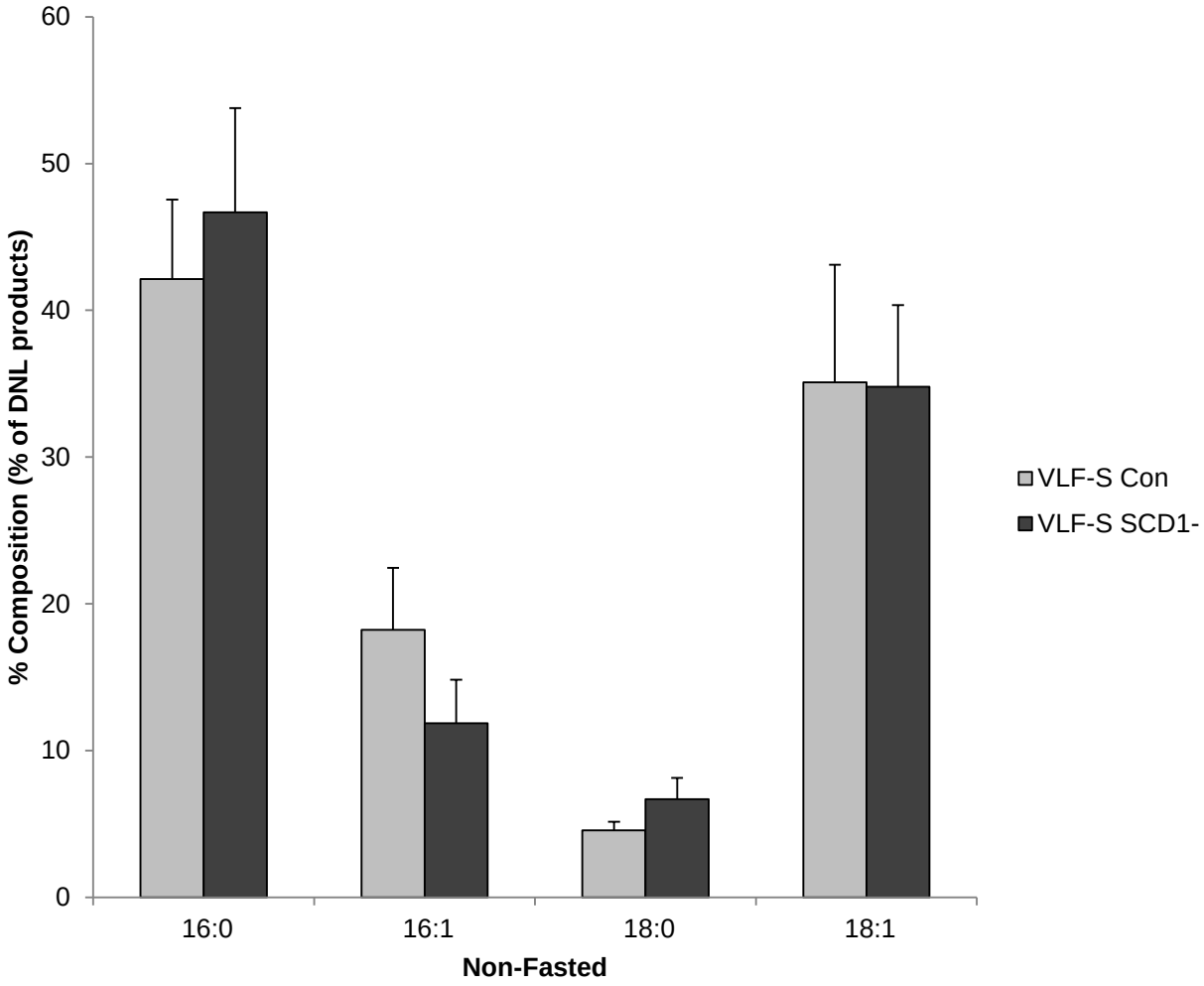
a) Muscle fatty acid composition – VLF-M Non-fasted group



b) Muscle fatty acid composition – VLF-M Fasted group



c) Muscle fatty acid composition – VLF-S Non-fasted group



d) Muscle fatty acid composition – VLF-S Fasted group

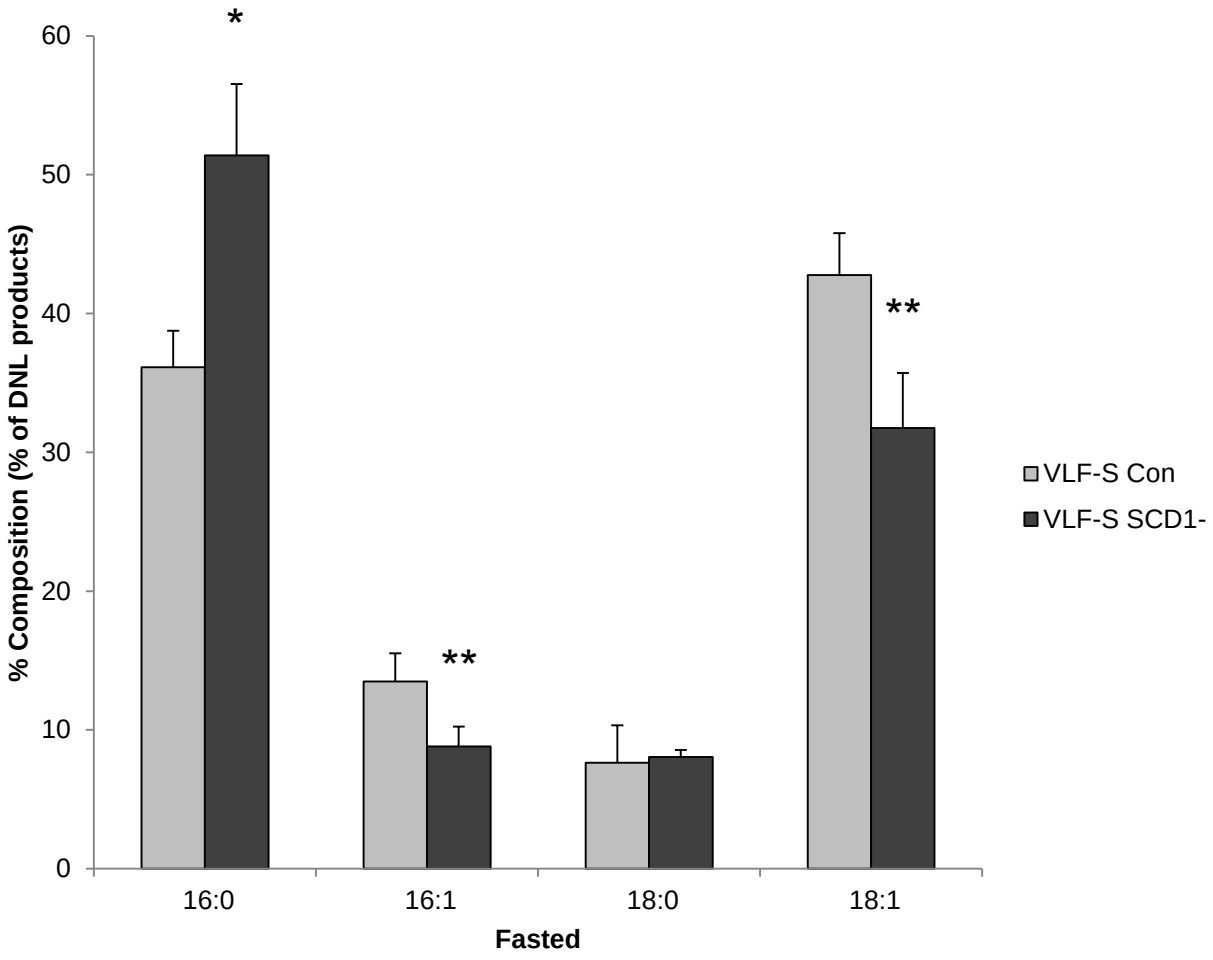


Figure 14. Muscle TG – % of DNL products.
VLF-M Non-fasted (a), VLF-M Fasted (b), VLF-S Non-fasted (c), VLF-S Fasted (d) Values are expressed as mean ± SEM (n=8/group). Statistical significance assessed at $p < 0.05$, indicated as *; $p > 0.05$ indicate as **.

Chapter 5: DISCUSSION

5.1 Summary of Findings

This study establishes an insight into the functional role of SCD1 activity in normal lipid partitioning and the maintenance of a homeostatic balance between SFAs and MUFAs. Primary findings of this study investigating SCD1 inhibition in wild-type C57Bl6 mice are marked hypercholesterolemia, downregulated skeletal muscle nutrient handling protein content, and increased hepatic and muscular SFA composition, however the molecular controls of skeletal muscle protein translation via mTOR activation remained unaltered.

5.2 Primary Findings

5.2.1 Effect of high carbohydrate very-low fat (HCVLF) diet

The HCVLF diet that was administered in this study was used to maximize endogenous lipid production in order to examine how changes in SCD1 activity affect endogenous oleate, lipid partitioning and cell signaling. This diet typically induces SCD1 activity and other lipogenic genes through the abundance of glycolytic derivatives, primarily acetyl-CoA; in order to produce MUFAs for the biosynthesis of lipids. This study acutely downregulated SCD1 expression and the HCVLF diet in wild-type C57Bl6 mice enabled us to analyze exacerbated systemic effects of reduced endogenous oleate with minimal interference from MUFAs from dietary sources as well as interference from

genetic modification. Thus, the metabolic effects this diet had on the mice must be differentiated from effects of SCD1 inhibition. Firstly, this HCVLF diet is not an obesogenic diet in C57Bl6 mice as our mice exhibited a normal phenotype, as opposed to HF diets, where mice fed a HF diet have significant increases in fat volume, fat size and a myriad of other metabolic effects associated with obesity (Sumiyoshi *et al*., 2006). Secondly, both dietary treatment groups exhibited low plasma TG values, which is also attributed to the HC diet as it is reported that C57Bl6 mice that are fed a HC diet, particularly high in sucrose, remain lean and demonstrate significantly lower TG concentrations than HF-fed mice, which correlates closely to their low-fat low-sucrose littermates. Collectively, these reports indicate that a considerable amount of fat is required in the C57Bl6 mouse diet to induce a state of obesity and its biochemical effects and appears to be a genetic trait of this strain (Surwit *et al*., 1995). In addition, HC diets, high in simple carbohydrates, are known to induce hyperglycemia, hyperinsulinemia and hypercholesterolemia compared to mice fed chow or purified AIN rodent diets, accounting for the higher than normal insulin values in our study (Sumiyoshi *et al*., 2006).

On the other hand, the VLF component of the diet largely contributed to the severe hepatic and systemic dysfunction in SCD1- mice, specifically the dyslipidemia, due to the fact that this diet eliminated the majority of the dietary oleate component and exacerbated any metabolic effects due to extremely low levels of both dietary and endogenous oleate. In contrast, if our mice were fed a standard chow regimen, the level to which SCD1 is induced for DNL would be much lower and the dietary fat component within standard chow would supply an adequate amount of dietary oleate to SCD1- mice

to prevent most if any severe metabolic effects seen in this VLF diet, making it more difficult to acutely study the metabolic role of SCD1.

5.2.2 Effect of SCD1 deficiency on plasma lipids and its implications

Following the acute inhibition of SCD1, severe dyslipidemia occurred with significantly increased TC and non-HDL cholesterol levels with a modest increase in HDL cholesterol. The acute increase in blood lipids observed in SCD1- mice is part of the complex phenotype created in response to SCD1 deficiency when combined with a HCVLF diet (Flowers *et al*., 2006). In comparison, mice with normal SCD1 function maintained relatively normal lipid levels whereas SCD1- mice and most interestingly, VLF-M SCD1- mice, demonstrated hypercholesterolemia indicating that dietary MUFAs are not sufficient in rescuing normal lipid values and that it is normal SCD1 activity which is required for cholesterol homeostasis. However, this effect on blood lipids does not seem to be replicated in all models of SCD1 inhibition, particularly those using ASO-mediated SCD1 inhibition in wild-type mice or atherosclerosis mouse models as well as longer phases of SCD1 inhibition (Brown *et al*., 2008). The hypercholesterolemia observed in our study is interesting as this effect on lipids in response to SCD1 inhibition in wild-type C576Bl6 mice is the first example of dyslipidemia with this particular SCD1 inhibitor, MF-438. However, as SCD1 inhibition was only in effect for 72 hours before termination, this observation may solely be an acute transient effect and dissipate following a longer term of SCD1 inhibition. Nevertheless, it is evident in our study that endogenous MUFAs produced via SCD1 activity is required for normal lipemia and given our wild-type mouse model, administration of an SCD1 inhibitor as an anti-obesity

therapy should be cautioned in order to prevent deleterious effects of dyslipidemia and progression of atherosclerosis.

5.2.3 Effect of acute SCD1 inhibition on skeletal muscle lipid handling

It was observed in this study that mice with depressed SCD1 function had significantly increased levels of plasma total and non-HDL cholesterol when compared to wild-type mice on the same diet, which is in agreement with previous reports (Flowers *et al*., 2006; Brown *et al*., 2008; Brown *et al*., 2010). The increase in circulating lipoproteins due to SCD1 inhibition occurred in the absence of rising TG levels. This observation correlates to the reduced levels of available endogenous MUFAs available to form TGs, which conforms to the model that SCD1 inhibition reduces DNL and creates a phenotype that is resistant to DIO.

As it has been implicated that endogenous MUFA levels play an integral role in cell signaling compared to dietary MUFAs, the effect that SCD1 inhibition has on endogenous MUFA levels may alter the function of many key elements involved in cholesterol metabolism, particularly transcription factors, enzymes, receptors and transport proteins. Hence, it is possible that the observed decrease in skeletal muscle protein expression of nutrient handling proteins, Lpl and CD36, may have contributed to the decreased clearance of circulating lipoproteins, which is further supported by a simultaneous decrease in plasma lipoprotein lipase. In support of this hypothesis, a link was established between decreased Lpl activity and delayed clearance of TG-rich lipoproteins and decreased HDL cholesterol levels in a case-control study using offspring of myocardial infarction (MI) patients (Lekhal *et al*., 2008).

Moreover, total protein content of both Lpl and CD36 was found to be significantly reduced in instances of increased saturation, particularly in VLF-M SCD1- and both VLF-S CON and VLF-S SCD1- mice, all of which either have very low levels of dietary or endogenous oleate. The decreased expression of these proteins indicates a reduction in the hydrolysis of lipoproteins into their constituents as well as a reduction in the transport of these fatty acids for cellular use. While in this study we did not measure, we speculate that situations that create an influx of SFAs, either from diet or endogenous synthesis, downregulate the activity these nutrient handling proteins in the effort to prevent cellular lipotoxicity, which is associated with various metabolic disorders including insulin resistance and inflammation (Dresner *et al*., 1999; Kashyap *et al*., 2004; Richardson *et al*., 2005). In a study by Holland et al, it was reported that saturated lard fat-based emulsions increased tissue ceramide levels and insulin resistance compared to unsaturated soybean-based emulsions (Holland *et al*., 2007). For instance, ceramide is one lipotoxic molecule that is thought to cause abnormal cell signaling contributing to the progression of insulin resistance. Moreover, in models of cardiac myocyte Lpl knockout mice, it was shown to lead to myocardial dysfunction causing heart failure with hypertension, whereas, in mice models of overexpressed cardiomyocyte-anchored Lpl created cardiac lipotoxicity due to ceramide accumulation (Lee & Goldberg, 2007). These reports deduce that the activity of Lpl is highly regulated and acts as a gatekeeper for fatty acids potentially either creating a state of dyslipidemia with decreased Lpl activity or allowing a flood of fatty acids into the cell leading to fat storage and eventual lipotoxicity with overexpressed Lpl activity. Furthermore, in a mouse model of Lpl-deficiency, it was evident that along with hypertriglyceridemia including increased levels

of TC, these mice exhibited accelerated development of atherosclerotic lesions in the aortic sinuses as well as fatty streaks that contained foam cells (Zhang *et al*., 2008). The results from this study show numerous similarities to prior reports using SCD1-deficient mice (Brown *et al*., 2008) but also show results analogous to our findings, particularly the link between downregulated Lpl protein and dyslipidemia. Therefore, overexpression and underexpression of Lpl activity is associated with the progression of two chronic diseases, insulin resistance and type 2 diabetes and atherosclerosis, respectively. Unlike previous reports, this study observes dyslipidemia with SCD1 inhibition for the first time in wild-type C57BL6 mice as opposed to genetically modified mice, particularly atherosclerosis-prone $LDLr^{-/-}$ $ApoB^{100/100}$ mice, which may already come with a host of contributing factors to increases in lipoprotein levels besides the effects of SCD1 inhibition.

5.2.4 Effect of acute SCD1 inhibition on protein phosphorylation of the Akt/mTOR/S6k pathway

To determine the origin of the resistance to obesity exhibited in SCD1- mice most commonly purported to be an increase in energy expenditure, we studied changes in skeletal muscle phosphorylation of key proteins within the Akt/mTOR/S6K pathway that lead to protein translation and muscle cell growth. We observed a significant increase in phosphorylation of intermediate protein Akt in VLF-S SCD1- fasted group but unexpectedly observed no significant changes in phosphorylation of any other signaling protein of the pathway. Interestingly, increased Akt phosphorylation in the VLF-S SCD1- mice did not trigger an increase in phosphorylation of its downstream targets as

expected, however, this observation may suggest that there are other regulators of Akt and may be activated independently of upstream and downstream target proteins in SCD1- mice (Wang *et al*., 2009). In congruence with this observation, a similar conclusion was found by Wang et al, where $SMLPL^{-/-}$ mice were found to have increased skeletal muscle insulin sensitivity but showed no changes in IRS1 phosphorylation even though insulin-stimulated Akt phosphorylation was increased. This observation led them to conclude that the altered Lpl-derived lipids in $SMLPL^{-/-}$ affect Akt activation independently from IRS-1 and that the increase in skeletal muscle insulin sensitivity was likely due to lower muscle TG levels (Wang *et al*., 2009). Moreover, although IRS1 and Akt are normally activated via insulin signaling, mTOR can be activated via alternate nutrients, such as fatty acids and amino acids, however, it appears that mTOR and its downstream target proteins also were not activated in this matter indicating that nutrients within the skeletal muscle cell may be abnormally regulated in response to SCD1 inhibition and dietary treatment.

Since it was observed that the phosphorylation of key cell signaling proteins within the Akt/mTOR/S6K network were not different across treatments, it may be concluded that skeletal muscle protein translation remains unchanged in response to acute SCD1 inhibition and that the observed increase in energy expenditure originates from alternate mechanisms and may not be due to increased protein synthesis and lean muscle production.

5.2.5 Effect of acute SCD1 inhibition on skeletal muscle protein expression of key nutrient handling proteins

To determine whether changes in phosphorylation of key proteins within the Akt/mTOR/S6K protein translation pathway were linked to a modification in the handling of various nutrients that activate the insulin signaling pathway, the expression of Lpl and CD36 was examined. Interestingly, although no significant changes in protein phosphorylation within the Akt/mTOR/S6K pathway, it was observed that protein content of Lpl and CD36 were significantly decreased in response to SCD1 inhibition in both dietary treatments, VLF-M in the non-fasted state and VLF-S in the fasted state. This observation indicates that although expression of nutrient handling proteins was altered, this action had no significant effect on the activation within the Akt/mTOR/S6K protein translation pathway.

Although no link was made between mTOR activation and nutrient handling, the decrease in skeletal muscle protein content of Lpl and CD36 across both dietary treatments signifies that the function of these proteins are downregulated and that the lipoprotein-derived fatty acids are being hydrolyzed and oxidized to a lesser rate in muscle. As skeletal muscle Lpl activity responds to a variety of physiological changes, particularly fasting and feeding, Lpl activity is known to remain unchanged or decrease following a 12-hr fast. Moreover, skeletal muscle Lpl activity has also been shown to be decreased after consumption of a HC diet (70% of energy) due to the effect of insulin action on glucose uptake and oxidation, dampening FFA oxidation and promoting lipid storage (Ferland *et al*., 2011). Therefore, the consumption of a HC diet for VLF-M and the fasted state of VLF-S along with the postulated increase in skeletal muscle SFAs from

SCD1 inhibition may all be contributing to the significant decrease in Lpl activity in the respective mice.

As previously mentioned in Section 5.2.3, the decreased protein expression of Lpl and CD36 may be creating the heightened lipoproteinemia in effort to prevent cellular lipotoxicity due to an increased level of SFAs. The differential change in expression seen across energy states may be related to a proportional change in the SFA component of the composition in response to acute SCD1 inhibition. Although the decreased content of Lpl expressed in SCD1- mice may be contributing to the dyslipidemia, it also may be playing a protective role preventing the lipolysis of saturated-rich lipoproteins and probable lipotoxicity. Although lipotoxicity is generally correlated to an increase in intracellular TGs, SCD1- mice exhibit no change in TGs as well as no increase in weight. While we did not measure, we speculate that SCD1- mice may have increased levels of unoxidized derivatives of palmitoyl-coA due to the inability to convert palmitoyl-coA to palmitate, with ceramide as the most likely contributing lipotoxin. Therefore, Lpl protein levels may be decreasing in response to the unoxidized saturated derivatives of SCD1 inhibition so as to regulate the hydrolysis and uptake of highly saturated lipoproteins derivatives. This in turn would be reducing the substrates for *de novo* ceramide synthesis, preventing increasing ceramide levels, possible lipotoxicity and normalizing insulin response. In addition, previous reports have shown that genetic SCD1 knockout mice also show reduced ceramide levels in oxidative muscle tissues, however the current mechanism linked is a downregulation in the activity of SPT, an enzyme active in the initial step of *de novo* ceramide synthesis (Dobrzyn *et al*., 2005a). Thus, the effect of decreased Lpl protein content seen in our mice together with the downregulated SPT

activity observed in SCD1$^{-/-}$ mice both may be contributing to reduced tissue ceramide levels and the increased insulin sensitivity and hypoglycemia consistently shown in SCD1$^{-/-}$ mice (Miyazaki *et al*., 2007; Dobrzyn *et al*., 2005a).

Due to the effect of skeletal muscle Lpl protein content and the decreased lipoprotein hydrolysis and resultant FFAs available for transport in VLF-M and VLF-S SCD1- mice, a significant decrease in skeletal muscle CD36 protein content was observed in the same mice indicating a reduction in the transport of fatty acids for oxidation in muscle. However, several reports have suggested that mitochondrial CD36, not whole CD36 protein, is responsible for final fatty acid oxidation as whole CD36 protein is necessary to transport fatty acids across plasma membrane but only mitochondrial CD36 is accountable for fatty acid translocation to the mitochondria in which fatty acid oxidation occurs and is regulated (Bonen *et al*., 2004; Holloway *et al*., 2008). Therefore, the decrease in skeletal muscle whole CD36 protein content is likely a downstream effect of the attenuated Lpl protein expression causing a reduced abundance of lipoprotein-derived fatty acids and thus fewer fatty acids to transport for oxidation.

These proteins also play an important role in partitioning lipid nutrients towards adipose tissue for storage or skeletal muscle for oxidation, and thus, changes in skeletal muscle expression of these proteins may possibly be a source of the increased energy expenditure seen in response to SCD1 inhibition (Wang *et al*., 2009). However, since the level of skeletal muscle Lpl was shown to be decreased in SCD1- mice in both dietary treatments, it is plausible to suggest that TG-rich lipoproteins are not being hydrolyzed by skeletal muscle Lpl and transported by muscle CD36 as readily and thus, theoretically should be shuttled towards storage rather than oxidation with a simultaneous increase in

adipose tissue Lpl protein expression. However the lean phenotype of SCD1- mice and resistance to obesity does not agree with this theory and suggests that the increase in energy expenditure is originating from alternate mechanisms, organs or tissues.

5.2.6 Effect of acute SCD1 inhibition on liver and muscle tissue fatty acid composition

To confirm whether the decreased expression in Lpl and CD36 protein observed in SCD1- mice was in effort to prevent cellular lipotoxicity from accumulation of SFAs, the fatty acid composition of both liver and skeletal muscle was examined. It was found that overall levels of SFAs, particularly palmitate (16:0) and stearate (18:0), are increased and MUFAs, palmitoleate (16:1) and oleate (18:1), are decreased in all SCD1- mice in both energy states. This degree of greater saturation in liver and muscle indicates that this shift in saturation is likely due to the decrease in hepatic desaturation index due to SCD1 inhibition and proportionate inability to create MUFAs. In comparison, previous reports using SCD1 ASO treatment have also observed significant enrichment of SFAs regardless of diet in plasma lipids and macrophage membranes causing accelerated atherosclerosis, which also could not be rescued by the VLF-M diet, or oleate, as in our study (Brown *et al*., 2008). Briefly, we believe that acute hepatic SCD1 inhibition results in TGs that are highly saturated due to the inability to desaturate 16:0 and 18:0 into their monounsaturated counterparts, 16:1 and 18:1. The saturated TGs are then converted into VLDL particles with a highly saturated TG core, further giving rise to highly saturated LDL particles. These lipoproteins are hydrolyzed by Lpl at a decreased rate in SCD1- mice to restrict the uptake of SFAs into the muscle cell, which also shows decreased

SCD1 expression, however accumulation of SFAs in muscle tissue is still evident. Interestingly, significant accumulation of SFAs, namely 16:0, with simultaneous significant reduction of MUFAs, 16:1 and 18:1, was only observed in the fasted state of VLF-S SCD1- mice muscle. This observation may indicate dietary oleate as well as sufficient energy may rescue significant over accumulation of SFAs in muscle as these groups exhibited increased SFA accumulation, however, to a less significant extent.

A plausible explanation for why VLF-M and nonfasted groups did not exhibit significant accumulation of SFAs is that these mice may have been supplied with enough MUFAs from their diet and energy state to package TGs that were slightly less saturated than their VLF-S and fasted counterparts and thus resulted in proportionately less saturated fat accumulation in muscle tissue. Furthermore, it is within fasted VLF-S SCD1- mice that Lpl and CD36 protein content is significantly attenuated, which corroborates with our fatty acid results in muscle and adds to the notion that these observations are suggested to be in response to greater saturation of lipoprotein particles.

It is a well-documented concept that SFAs are strong proinflammatory molecules and prior studies also using mice lacking SCD1 have confirmed this notion that downregulated hepatic SCD1 exacerbates inflammation and inflammation-driven diseases, particularly colitis and atherosclerosis, due to a notable accumulation of SFAs in various tissues, including liver, aorta and macrophages (Nicholls *et al*., 2006; Chen *et al*., 2008; Brown *et al*., 2008). Moreover, non-fasted VLF-M and VLF-S SCD1- mice developed a degree of liver inflammation as demonstrated increased levels of plasma hepatic inflammatory markers, alanine transaminase (ALT) and aspartate transaminase (AST) (data shown in Appendix 6 & 7). Therefore, it suggests that normal expression of

SCD1 and its function of producing endogenous MUFAs, 18:1 and 16:1, play a crucial role in protecting against accumulation of SFAs, particularly in the liver, where dietary oleate was not sufficient in rescuing hepatic accumulation of 18:0. Whereas in the muscle, it appears that various regulatory mechanisms, such as nutrient handling proteins Lpl and CD36, may modify their expression accordingly to provide cellular protection from detrimental effects of SFA accumulation, but also responded favorably to dietary oleate consumption as significant accumulation of saturates did not result in VLF-M SCD1- mice.

Overall, it has been shown that normal SCD1 expression acts to prevent overaccumulation of SFAs through its natural enzymatic conversion of SFAs to MUFAs, which inadvertently also acts to suppress proinflammatory signaling and protect against inflammation-driven diseases, such as atherosclerosis, rheumatoid arthritis and inflammatory bowel disease.

5.2.7 Effect of acute SCD1 inhibition on circulating glucose and insulin levels

Previous studies have observed that SCD1- mice exhibit increased insulin sensitivity and normalized glucose levels in mice and the proposed mechanisms behind these effects are modified insulin signaling components, particularly downregulated mRNA levels, protein mass and activity of protein-tyrosine phosphatase -1B and elevated phosphorylation of IR, IRS1, IRS2,Akt (Ser-473) and Akt (Thr-308) (Rahman *et al*., 2003). Our study also exhibited decreased plasma glucose levels with unchanged insulin levels in VLF-S SCD1- mice, however, unlike the Rahman et al report, it is observed that this effect may relate back to lipid partitioning, nutrient handling and effects on insulin

response due to intramuscular lipid content. For instance, levels of phosphorylated Akt (Ser-473) were elevated in VLF-S SCD1- but did not confer a direct increase in the protein content of GLUT4 and subsequent glucose uptake, indicating that GLUT4 activity is not responsible for the hypoglycemia seen in SCD1- mice. In fact, GLUT4 protein content was significantly attenuated in response to acute SCD1 inhibition signifying a probable reduction in basal glucose uptake at the skeletal muscle. This observation suggests that the glucose available is being abnormally disposed throughout the body creating the observed hypoglycemia, as it cannot be attributed to the skeletal muscle as the typical site for glucose disposal. In addition to the downregulated skeletal muscle GLUT4 content, there was no identifiable changes in phosphorylation of IRS1, the protein responsible for initiating downstream PI3K/Akt pathways from insulin receptor signal transmission, from which GLUT4 activity is regulated. The absence of change in IRS1 phosphorylation is likely due to the uniform insulin levels across treatments, which provides an explanation as to why GLUT4 protein activity did not increase. Alternatively, the decrease in GLUT4 muscle protein content in VLF-S SCD1- mice may signify that glucose is being utilized by an alternate tissue, such as adipose tissue, and thus contributing to the decreased glycemia in these mice.

Moreover, this observation indicates that levels of endogenous oleate together with dietary oleate may help to regulate glycemia in a state of glucose overload as SCD1 inhibition exhibited decreased plasma glucose levels in both dietary treatments but were decreased to a significantly greater extent in VLF-S mice. Alternatively, the decrease in glycemia in VLF-S SCD1- mice may reflect a shift in insulin sensitivity due to

unchanged circulating insulin levels as shown in prior studies (Rahman *et al*., 2003; Miyazaki *et al*., 2007).

5.3 Strengths and Limitations

5.3.1 Strengths

This study is the first of our knowledge to investigate the effects of acute SCD1 inhibition using the triazole-based small molecule, MF-438 on lipid metabolism in wild-type mice with the intent to examine downstream effects in peripheral tissues, namely soleus muscle, on nutrient handling and protein translation via mTOR activation. Several studies have demonstrated effects of SCD1 inhibition on skeletal muscle mTOR activation but have always been reported as a link to the observed increase in insulin sensitivity with no mention of protein translation and possible cell growth as a source for increased energy expenditure and resistance to weight gain (Dobrzyn *et al*., 2010; Voss *et al*., 2005; Rahman *et al*., 2003; Rahman *et al*., 2005). In addition, there is a significant void in research of SCD1 inhibition within animal models free of some form of genetic modification, whether it is a SCD1 knockout mouse or an atherosclerotic mouse with tampered cholesterol metabolism, which may present noteworthy results but may stream from underlying metabolic effects of the modified animal strain. The notion that our study used wild-type C57Bl6 mice provides an understanding of the effects of SCD1 inhibition in a model with prior normal SCD1 function, providing an outlook of SCD1 inhibition as an obesity therapy, without the interference of genetic modification.

Therefore, this study presents effects of acute SCD1 inhibition from a variety of new angles for the first time that will vastly contribute to our knowledge of SCD1 inhibition as well as present new avenues from which to continue research.

5.3.2 Limitations

There are several limitations of this study. First and most importantly, since SCD1 was only inhibited for an acute period of 72 hours, the effects of long term inhibition are unknown in our model and present the question of whether any of the effects demonstrated in our study would dissipate or be exacerbated following a longer inhibition period. For instance, changes in blood lipids observed in longer-term SCD1 inhibition are not as severe as the dyslipidemia demonstrated in this study suggesting that dramatic changes in blood lipids are an acute effect and do not translate to longer lengths of inhibition. Similarly, the acute increase in hepatic SFA might also require a longer period of time to metabolically adjust to the lack of MUFA production and buildup of SFAs and cope by increasing fatty acid oxidation in order to regain balance.

5.4 Implications

This study exemplifies in a highly intricate manner that interfering with the normal function of a highly regulated element in lipid metabolism, SCD1, may have noteworthy therapeutic effects on a target metabolic condition of obesity and insulin resistance but may extend further having profound metabolic outcomes in blood and tissues that were not anticipated and may be more deleterious than the condition in which

was to be mitigated. For instance, research has shown that SCD1 is an enhancer of MetS and that inhibition of this enzyme severely impairs lipid biosynthesis, reducing MUFA and TG production resulting in increased energy expenditure and improved glucose tolerance, thereby creating a model for resistance to DIO and insulin resistance. However, in addition to these replicated results of SCD1 inhibition, our data demonstrate that SCD1 inhibition creates significant accumulation of SFAs (16:0 and 18:0) in both the liver and skeletal muscle as well as considerable dyslipidemia, to which consumption of dietary MUFAs could not rescue and both are risk factors for inflammation and atherosclerosis. These effects may be a result of the significant reduction in expression of nutrient handling proteins, Lpl and CD36, which may have been a response to protect muscle cells from SFA accumulation and in doing so contributed to the observed decrease in plasma lipoprotein clearance. Therefore, although SCD1 inhibition may provide protection against development of MetS it also presents effects that may promote atherosclerosis requiring additional studies to investigate further into these undesirable effects.

This study also demonstrates the far-reaching effects that different levels of SCD1 function can have on a systemic level, which suggests that natural variations in the SCD1 gene may express these phenotypes accordingly. This notion is in line with a human report by Warensjo et al, where polymorphisms of the SCD1 gene were associated with body fat distribution and insulin sensitivity that reflect closely with animal data (Warensjo *et al*., 2007). This report along with our data suggests that SCD1 acts as an important lipid gatekeeper regulating the degree of predisposition to obesity, insulin resistance and atherosclerosis that its activity is associated.

Overall, the role that SCD1 activity plays in human metabolism will remain to be a subject of interest in the field of obesity research as its multi-faceted properties are yet to be completely understood, however, extensive progress in comprehending the metabolic role of SCD1 has been made over the last 10 years contributing to many advancements in our knowledge of lipid, glucose and skeletal muscle metabolism.

5.5 Conclusion

From a scientific perspective this study has improved our understanding of the consequences of interfering with the body's normal fat production mechanisms. Collectively, the present data demonstrates that acute SCD1 inhibition in wild-type C57Bl6 mice fed a lipogenic diet creates severe hypercholesterolemia due to modified nutrient handling protein activity in response to significant SFA tissue accumulation due to reduced endogenous MUFA production, all of which was not prevented by dietary MUFAs. Our results show that MUFAs produced in the liver behave differently than dietary MUFAs and are essential for normal nutrient handling by other tissues and that interfering with fat production in the liver has whole body consequences. Overall, SCD1 activity and its role in DNL imparts a regulatory balance in the cellular SFA: MUFA ratio which as shown in our study and previous reports can convey significant metabolic benefits and consequences either contributing to prevention or progression of chronic disease.

Chapter 6: FUTURE WORK

Future work of exploring further into the lipid effects of SCD1 inhibition on peripheral tissues and to further investigate the origin for increased energy expenditure in our model would be to examine ceramide and AMPK levels, respectively, to verify if results for these parameters demonstrated in previous reports of SCD1 inhibition using alternate models are replicated using wild-type mice fed a HCVLF diet. A short preamble as to the relevance of this future work in SCD1 inhibition and lipid metabolism is reviewed here.

6.1 SCD1 Inhibition and the relationship between ceramide levels on insulin sensitivity and beta oxidation

It has been proposed that an overaccumulation of lipids in nonadipose tissues disrupts normal cell signaling and function through lipotoxicity leading to manifestations of the MetS and diabetes, however, recent reports have suggested that the lipid-induced damage to tissues is triggered via by-products of unoxidized acyl-coAs, primarily ceramide (Unger *et al*., 2003). Increased ceramide levels have been implicated in the development of insulin resistance as glucose uptake is blunted in muscle cells due to downregulated Akt activation and impaired GLUT4 translocation (Summers *et al*., 1998). In addition, heightened levels of ceramide have been shown to contribute to lipotoxicity of the heart and pancreatic β-cells and are necessary for the development of palmitic-acid induced insulin resistance (Shimabukuro *et al*., 1998; Zhou *et al*., 2000). The signaling pool of ceramide is a product of *de novo* ceramide synthesis and/or sphingomyelin

hydrolysis and a loss of SCD1 function has demonstrated a decrease in ceramide levels by modifying elements of both these processes. Endogenous ceramide is produced initially by the esterification of palmitoyl-coA and serine, which is catalyzed by the enzyme SPT and marks the beginning of *de novo* synthesis. It has been shown that a deficiency of SCD1 causes a decrease in this process due to a downregulation in the activity of SPT and the expression of both its subunits, thus exhibiting a decrease in total ceramide levels in oxidative soleus and red gastrocnemius muscle tissue by almost 50% (Dobrzyn *et al.*, 2005a). This decrease in SPT activity may be in response to a decreased availability of substrate for *de novo* ceramide synthesis, since FA-CoAs are primary factors for endogenously produced ceramide and both FFAs and FA-CoAs are significantly reduced in muscle with SCD1 inhibition due to decreased endogenous oleate (Dobrzyn *et al*., 2005a). In addition to modulating *de novo* synthesis, a loss of SCD1 function also appears to affect ceramide levels in muscle tissues, however to a lesser extent, by decreasing the sphingomyelin content in soleus and red gastrocnemius muscle tissue. However, gene expression or activities of sphingomyelinases in these tissues seem to remain unchanged concluding that SCD1 inhibition does not regulate the rate of ceramide formation via sphingomyelin hydrolysis despite lower sphingomyelin content in $SCD1^{-/-}$ mice.

Most interestingly, the decrease in ceramide content was shown to be less pronounced in white gastrocnemius muscle, a glycolytic tissue, as opposed to oxidative soleus and red gastrocnemius muscles which showed a decreased in ceramide content by almost 50%, therefore concluding that the primary reason for decreased ceramide content in $SCD1^{-/-}$ mice originates from an increase in beta oxidation. In congruence with this

observation on ceramide content, the same effect is seen with AMPK phosphorylation in $SCD1^{-/-}$ mice, where phosphorylation is increased in oxidative muscle but remains unchanged in glycolytic tissues enforcing the link of beta oxidation to these effects. However, how does SCD1 inhibition and the increased level of beta oxidation both lead to an increase in AMPK phosphorylation as well as decreased ceramide content, is one responsible for the other or are they parallel effects?

6.2 AMP-activated protein kinase activation and energy expenditure

AMPK is a heterotrimeric enzyme that acts as a metabolic sensor for cellular energy stores and regulates gene expression of various lipid synthesis and lipid oxidation enzymes via phosphorylation of a series of transcription factors and coactivators. AMPK is proposed to influence lipid metabolism from a number of mechanisms and SCD1 inhibition acts to activate AMPK leading to many of the metabolic changes observed.

Of primary interest is the role of AMPK in the increase of energy expenditure in SCD1-deficient mice and it has been shown in a study by Dobrzyn and colleagues that mice with targeted disruption of SCD1 have increased hepatic activation of AMPK (Dobrzyn *et al*., 2004). The mechanism by which AMPK acts to increase beta oxidation is firstly by phosphorylating and deactivating ACC at Ser-79. Inhibition of ACC activity by AMPK activation subsequently reduces the content of malonyl-coA, whereby a halt in malonyl-coA production acts to release the repressive action of malonyl-coA on the activity of CPT1. CPT1 shuttles palmitate into mitochondria to be oxidized by way of the mitochondrial CPT shuttle system and through AMPK activation, palmitate oxidation

is increased and hence, energy expenditure as well (Kerner & Hoppel, 2000). The study by Dobrzyn and colleagues investigated the role of AMPK activation in response to SCD1 inhibition and showed that as hypothesized AMPK activation is increased in SCD1-deficient mice. In addition, phosphorylation of ACC1 and ACC2 protein was significantly increased, whereby ACC1 and ACC2 protein levels are reduced, which reflects a decrease in ACC mRNA. Consequently, malonyl-coA content was also significantly decreased in this study which led to the expected increase in mitochondrial fatty acid oxidation as CPT1 protein levels were increased allowing for upregulated fatty acid transport into the mitochondria for beta oxidation.

In addition, AMPK activation was also tested in the skeletal muscle of SCD1 GKO mice, of which soleus, red gastrocnemius and white gastrocnemius muscle was examined (Dobrzyn *et al*., 2004). In congruence with the previously mentioned work by Dobzryn and colleagues, this study, also by Dobrzyn and colleagues showed that SCD1-deficient mice also have increased phosphorylation in the α-subunits of AMPK in soleus and red gastrocnemius muscle, but not in white gastrocnemius muscle, due the glycolytic nature of white muscle and the oxidative nature of red muscle tissue (Dobrzyn *et al*., 2005b). Of particular interest is the work by Miyazaki and colleagues using the SCD1 LKO mice model and demonstrated that AMPK activation remains unchanged in the liver of this model, indicating that SCD1 must be globally absent in order to phosphorylate AMPK in the liver and the oxidative muscles (Miyazaki *et al*., 2007). However, since increased energy expenditure is also observed in the SCD1 LKO model and cannot be attributed to AMPK activation, the question arises of what and where is triggering the increase in energy expenditure in models besides SCD1 GKO?

AMPK activation also plays an important role in the reduced adiposity observed in SCD1-deficient mice related to TG synthesis. For instance, AMPK activation leads to the phosphorylation and deactivation of a key transcription factor in carbohydrate-induced lipid synthesis, ChREBP, resulting in downregulated expression of glucose-induced lipogenic genes, primarily pyruvate kinase, FAS and ACC1 (Woods *et al*., 2000). In addition, AMPK activation also depresses the expression of SREBP1c, a transcription factor that activates the induction of lipogenic genes responsible for fatty acid and TG synthesis in response to a diet high in lipids (Zhou *et al*., 2001). Therefore, mice with targeted disruption of the SCD1 gene that exhibit both an increase in energy expenditure and reduced fatty acid and TG synthesis may be originating from increased AMPK activation due to its metabolic influence on specific transcription factors as well as lipogenic enzymes that together impact whether energy is oxidized or stored as fat. However, the question still remains of what source is creating the increase in energy expenditure and reduced adiposity in mice that solely are SCD-deficient in the liver when it has been demonstrated AMPK activation contributes to this observation in SCD1 GKO mice but does not occur in SCD1 LKO mice, and thus may be originating from completely different mechanisms.

REFERENCES

Agatha, G., Hafer, R. & Zintl, F. (2001). Fatty acid composition of lymphocyte membrane phospholipids in children with acute leukemia. *Cancer Lett, 173*(2), 139-144.

Bergeron, K., Julien, P., Davis, T.A., Myre, A., & Thivierge, M.C. (2007). Long-chain n-3 fatty acids enhance neonatal insulin-regulated protein metabolism in piglets by differentially altering muscle lipid composition. *J Lipid Res, 48,* 2396-2410.

Binczek, E., Jenke, B., Holz, B., Günter, R.H., Thevis, M., & Stoffel, W. (2007). Obesity resistance of the stearoyl-CoA desaturase-deficient (scd1-/-) mouse results from disruption of the epidermal lipid barrier and adaptive thermoregulation. *Biol Chem, 388*(4), 405-418.

Bonen, A., Campbell, S.E., Benton, C.R., Chabowski, A., Coort, S.L.M., Han, X-X. et al. 2004. Regulation of fatty acid transport by fatty acid translocase/CD36. *Proc Nutr Soc, 63*(2), 245-249.

Bradford, M. (1976). A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal Biochem, 72,* 248-254.

Brown, J. M., Chung, S., Sawyer, J.K., Degirolamo, C., Alger, H.M., Nguyen, T., et al. (2008). Inhibition of stearoyl-coenzyme A desaturase 1 dissociates insulin resistance and obesity from atherosclerosis. *Circulation, 118,* 1467-1475.

Brown, J.M., Chung, S., Sawyer, J.K., Degirolamo, C., Alger, H.M., Nguyen, T.M., et al. (2010). Combined therapy of dietary fish oil and stearoyl-CoA desaturase 1 inhibition prevents the metabolic syndrome and atherosclerosis. *Arterioscler Thromb Vasc Biol, 30*(1), 24-30.

Chen, C., Shah, Y.M., Morimura, K., Krausz, K.W., Miyazaki, M., Richardson, T.A., et al. (2008). Metabolomics reveals that hepatic stearoyl-CoA desaturase 1 downregulation exacerbates inflammation and acute colitis. *Cell Metab*, *7*, 135–147.

Chevalier S., Marliss E.B., Morais J.A., Lamarche M., & Gougeon R. (2005). Whole-body protein anabolic response is resistant to the action of insulin in obese women. *Am J Clin Nutr,82*, 355–365.

Coiffier, E., Paris, R., & Lecerf, J. (1987). Effects of dietary saturated and polyunsaturated fat on lipoprotein lipase and hepatic triglyceride lipase activity. *Comp Biochem Physiol B, 88*(1), 187-192.

Corradetti, M.N. & Guan, K-L. (2006). Upstream of the mammalian target of rapamycin: do all roads pass through mTOR? *Oncogene, 25,* 6347-6360.

Davis, T.A., Fiorotto, M.L., Burrin, D.G., Reeds, P.J., Nguyen, H.V., Beckett, P.R., et al. (2002). Stimulation of protein synthesis by both insulin and amino acids is unique to skeletal muscle in neonatal pigs. *Am J Physiol Endocrinol Metab, 282*, E880–E890.

Dobrzyn, P., Dobrzyn, A., Miyazaki, M., Cohen, P., Asilmaz, E., Hardie, D.G. et al. (2004). Stearoyl-CoA desaturase 1 deficiency increases fatty acid oxidation by activating AMP-activated protein kinase in liver. *Proc Natl Acad Sci U S A, 101*(17), 6409-6414.

Dobrzyn, A., Dobrzyn, P., Lee, S-H., Miyazaki, M., Cohen, P., Asilmaz, E., et al. (2005a). Stearoyl-CoA desaturase-1 deficiency reduces ceramide synthesis by downregulating serine palmitoyltransferase and increasing β-oxidation in skeletal muscle. *Am J Physiol Endocrinol Metab, 288,* E599-E607.

Dobrzyn, A., Dobrzyn, P., Miyazaki, M., & Ntambi, J.M. (2005b). Polyunsaturated fatty acids do not activate AMP-activated protein kinase in mouse tissues. *Biochem Biophys Res Comm, 332,* 892-896.

Dobrzyn, P., Jazurek, M., & Dobrzyn, A. (2010). Stearoyl-CoA desaturase and insulin signaling - What is the molecular switch? *Biochim Biophys Acta, 1797*(6-7), 1189-1194.

Dresner A., Laurent D., Marcucci, M., Griffin, M.E., Dufour, S., Cline, G.W., et al. (1999). Effects of free fatty acids on glucose transport and IRS-1-associated phosphatidylinositol 3-kinase activity. *J Clin Invest, 103*(2), 253–259.

Ferland, A., Château-Degat, M-L., Hernandez, T.L. & Eckel, R.H. (2012). Tissue-specific responses of lipoprotein lipase to dietary macronutrient composition as a predictor of weight gain over 4 Years. *Obesity* doi: 10.1038/oby.2011.372.

Flowers, M.T., Groen, A.K., Oler, A.T., Keller, M.P., Choi, Y., Schueler, K.L., et al. (2006). Cholestasis and hypercholesterolemia in SCD1-deficient mice fed a low-fat, high-carbohydrate diet. *J Lipid Res, 47*(12), 2668-2680.

Flowers, M.T. & Ntambi, J.M. (2009). Stearoyl-CoA desaturase and its relation to high-carbohydrate diets and obesity. *Biochim Biophys Acta, 1791*(2), 85-91.

Foster, K.G., & Fingar, D.C. (2010). mTOR: Conducting the cellular signaling symphony. *J Biol Chem, 285*(19), 14071-14077.

Fujii, N., Jessen, N., & Goodyear, L.J. (2006). AMP-activated protein kinase and the regulation of glucose transport. *Am J Physiol Endocrinol Metab, 291*, E867-877.

Gingras, A-A., White, P.J., Chouinard, P.Y., Julien, P., Davis, T.A., Dombrowski, L., et al. (2007). Long-chain omega-3 fatty acids regulate bovine whole-body protein metabolism by promoting muscle insulin signalling to the Akt-mTOR-S6K1 pathway

and insulin sensitivity. *J Physiol, 579*(1), 269-284.

Gutierrez-Juarez, R., Pocai, A., Mulas, C., Ono, H., Bhanot, S., Monia, B.P., & Rossetti, L. (2006). Critical role of stearoyl-CoA desaturase-1 (SCD1) in the onset of diet-induced hepatic insulin resistance. *J Clin Invest, 116*, 1686–1695.

Han, X.X., Chabowski, A., Tandon, N.N., Calles-Escandon, J., Glatz, J.F., Luiken, J.J., et al. (2007). Metabolic challenges reveal impaired fatty acid metabolism and translocation of FAT/CD36 but not FABPpm in obese Zucker rat muscle. *Am J Physiol Endocrinol Metab, 293*(2), E566-E575.

Hara, K., Yonezawa, K., Weng, Q-P., Kozlowski, M.T., Belham, C., Avruch, J. (1998). Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. *J Biol Chem, 273*, 14484-14494.

Hardie, D.G. (2003). The AMP-activated protein kinase cascade: the key sensor of cellular energy status. *Endocrinology, 144,* 5179–5183.

Hardie, D.G., Hawley, S.A., & Scott, J.W. (2006). AMP-activated protein kinase - development of the energy sensor concept. *J Physiol, 574,* 7-15.

Hay, N. & Sonenberg, N. (2004). Upstream and downstream of mTOR. *Genes Dev, 18*, 1926-1945.

Heitman, J., Movva, N.R., & Hall, M.N. (1991). Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science, 253,* 905-909.

Hinault, C., Mothe-Satney, I., Gautier, N., & Van Obberghen, E. (2006). Amino acids require glucose to enhance, through phosphoinositide-dependent kinase 1, the insulin-activated protein kinase B cascade in insulin-resistant rat adipocytes. *Diabetologia, 49,* 1017-1026.

Holland, W.L., Brozinick, J.T., Wang, L-P., Hawkins, E.D., Sargent, K.M., Liu, Y. et al. (2007). Inhibition of ceramide synthesis ameliorates glucocorticoid-, saturated-fat-, and obesity-induced insulin resistance. *Cell Metab, 5,* 167-179.

Holloway, G.P., Bezaire V., Heigenhauser, G.J.F., Tandon, N.N., Glatza, J.F.C., Luiken, J.J.F.P., et al. (2006). Mitochondrial long chain fatty acid oxidation, fatty acid translocase/CD36 content and carnitine palmitoyltransferase I activity in human skeletal muscle during aerobic exercise. *J Physiol, 571*(1), 201-210.

Holloway, G.P., Luiken, J.J.F.P., Glatz, J.F.C., Spriet, L.L., & Bonen, A. (2008). Contribution of FAT/CD36 to the regulation of skeletal muscle fatty acid oxidation: an overview. *Acta Physiol, 194,* 293-309.

Jacinto, E., Loewith, R., Schmidt, A., Lin, S., Ruegg, M.A., Hall, A., & Hall, M.N. (2004). Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nat Cell Biol, 6,* 1122-1128.

Jeyapalan, A.S., Orellana, R.A., Suryawan, A., O'Connor, P.M.J., Nguyen, H.V., Escobar, J., et al. (2007). Glucose stimulates protein synthesis in skeletal muscle of neonatal pigs through an AMPK- and mTOR-independent process. *Am J Physiol Endocrinol Metab, 293,* 595-603.

Jiang, G., Li, Z., Liu, F., Ellsworth, K., Dallas-Yang, Q., Wu, M., et al. Prevention of obesity in mice by antisense oligonucleotide inhibitors of stearoyl-CoA desaturase-1. *J Clin Invest,* 115, 1030-1038.

Jones, B.H., Maher, M.A., Banz, W.J., Zemel, M.B., Whelan, J., Smith, P.J. & Moustaïd, N. (1996). Adipose tissue stearoyl-CoA desaturase mRNA is increased by obesity and decreased by polyunsaturated fatty acids. *Am J Physiol, 271,* E44-E49.

Kashyap, S.R., Belfort, R., Berria, R., Suraamornkul, S., Pratipranawatr, T., Finlayson, J., et al. (2004). Discordant effects of a chronic physiological increase in plasma FFA on insulin signaling in healthy subjects with or without a family history of type 2 diabetes. *Am J Physiol Endocrinol Metab, 287*(3), E537-E546.

Kerner, J., & Hoppel, C. (2000). Fatty acid import into mitochondria. *Biochim Biophys Acta, 1486,* 1-17.

Khamzina, L., Veilleux, A., Bergeron, S., & Marette, A. (2005). Increased activation of the mammalian target of rapamycin pathway in liver and skeletal muscle of obese rats: possible involvement in obesity-linked insulin resistance. *Endocrinology, 146,* 1473-1481.

Kim, J.K., Fillmore, J.J., Chen, Y., Yu, C., Moore, I.K., Pypaert, M., et al.(2001). Tissue-specific overexpression of lipoprotein lipase causes tissue-specific insulin resistance. *Proc Natl Acad Sci U S A, 98*(13), 7522-7527.

Kim, E. (2009). Mechanims of amino acid sensing in mTOR signaling pathway. *Nutrition Res Prac, 3*(1),64-71.

Kubica, N., Crispino, J.L., Gallagher, J.W., Kimball, S.R., & Jefferson, L.S. (2008). Activation of the mammalian target of rapamycin complex 1 is both necessary and sufficient to stimulate eukaryotic initiation factor 2Bε mRNA translation and protein synthesis. *Int J Biochem Cell Biol*, 40, 2522-2533.

Leclerc, J.P., Falgueyret, J.P., Girardin, M., Guay, J., Guiral, S., Huang, Z., et al. (2011). Conversion of systemically-distributed triazole-based stearoyl-CoA desaturase (SCD) uHTS hits into liver-targeted SCD inhibitors. *Bioorg Med Chem Lett, 21*(21), 6505-6509.

Lee, J., & Goldberg, I.J. (2007). Lipoprotein lipase-derived fatty acids: physiology and dysfunction. *Curr Hypertens Rep, 9*(6), 462-466.

Lekhal, S., Børvik, T., Nordøy, A. & Hansen, J.B. (2008). Decreased lipoprotein lipase activity and increased postprandial concentrations of triglyceride-rich lipoproteins in offspring of elderly survivors of myocardial infarction. *Nutr Metab Cardiovasc Dis, 18*(10), 700-6.

Li, C.S., Belair, L., Guay, J., Murgasva, R., Sturkenboom, W., Ramtohul, Y.K., et al. (2009). Thiazole analog as stearoyl-CoA desaturase 1 inhibitor. *Bioorg Med Chem Lett, 19*(17), 5214-5217.

Loewith, R., Jacinto, E., Wullschleger, S., Lorberg, A., Crespo, J.L., Bonenfant, D., et al. (2002). Two TOR complexes, only one of which is rapamycin-sensitive, have distinct roles in cell growth control. *Mol Cell, 10,* 457-468.

Martin, D.E., & Hall, M.N. (2005). The expanding TOR signaling network. *Curr Opin Cell Biol, 17,* 158-166.

Miyazaki, M., Kim, H.J., Man, W.C., & Ntambi, J.M. (2001). Oleoyl-CoA is the major de novo product of stearoyl-CoA desaturase 1 gene isoform and substrate for the biosynthesis of the Harderian gland 1-alkyl-2,3-diacylglycerol. *J Biol Chem, 276,* 39455-39461.

Miyazaki, M., Flower, M.T., Sampath, H., Chu, K., Otzelberger, C., Liu, X., & Ntambi, J.M. (2007). Hepatic stearoyl- CoA desaturase-1 deficiency protects mice from carbohydrate-induced adiposity and hepatic steatosis. *Cell Metab, 6,* 484–496.

Morrison, W.R. & Smith, L.M. (1964). Preparation of Fatty Acid Methyl Esters and Dimethylacetals from Lipids with Boron Fluoride – Methanol. *J Lipid Res, 5,* 600-608.

Mothe-Satney, I., Gautier, N., Hinault, C., Lawrence, J.C.L., & Van Obberghen, E. (2004). In rat hepatocytes glucagons increases mammalian target of rapamycin phosphorylation on serine 2448 but antagonizes the phosphorylation of its downstream targets induced by insulin and amino acids. *J Biol Chem, 279,* 42628-42637.

Nicholls, S.J., Lundman, P., Harmer, J.A., Cutri, B., Griffiths, K.A., Rye, K.A., et al. 2006. Consumption of saturated fat impairs the anti-inflammatory properties of high-density lipoprotein and endothelial function. *J Am Coll Cardiol, 48*(4), 715-720.

Nobukuni, T., Joaquin, M., & Roccio, M. (2005). Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. *Proc Natl Acad Sci USA, 102,* 14238–14243.

Ntambi, J.M., Miyazaki, M., Stoehr, J.P., Lan, H., Kendziorski, C.M., Yandell, B.S., et al. (2002). Loss of stearoyl-CoA desaturase-1 function protects mice against adiposity. *Proc Natl Acad Sci USA, 99*, 11482-11486.

O'Connor, P.M., Bush, J.A., Suryawan, A., Nguyen, H.V., & Davis, T.A. (2003). Insulin and amino acids independently stimulate skeletal muscle protein synthesis in neonatal pigs. *Am J Physiol Endocrinol Metab, 284*, E110–E119.

Pan, D.A., Hulbert, A.J. & Storlein, L.H. (1994). Dietary fats, membrane phospholipids and obesity. *J Nutr, 124*(9), 1555-1565.

Pettegrew, J.W., Panchalingam, K., Hamilton, R.L. & McClure, R.J. (2001). Brain membrane phospholipid alterations in Alzheimer's disease. *Neurochem Res, 26*(7), 771-782.

Proud, C.G. (2004). mTOR-mediated regulation of translation factors by amino acids. *Biochem Biophys Res Commun, 313*, 429-436.

Rahman, S.M., Dobrzyn, A., Dobrzyn, P., Lee, S-H., Miyazaki, M., & Ntambi, J.M. (2003). Stearoyl-CoA desaturase 1 deficiency elevates insulin-signaling components and down-regulates protein-tyrosine phosphatase 1B in muscle. *Proc Natl Acad Sci USA, 100(19),* 11110-11115.

Rahman, S.M., Dobrzyn, A., Lee, S-H., Dobrzyn, P., Miyazaki, M., & Ntambi, J.M. (2005). Stearoyl-CoA desaturase 1 deficiency increases insulin signaling and glycogen accumulation in brown adipose tissue. *Am J Physiol Endocrinol Metab 288*, E381-E387.

Reynolds, T.H. IV, Cinquino, N., Anthony, M., Phelphs, C.B., & Berk, E.Z. (2009). Insulin resistance without elevated mammalian target of rapamycin complex 1 activity in muscles of mice fed a high-fat diet. *J Appl Physiol, 107,* 1479 –1485.

Richardson, D.K., Kashyap, S., Bajaj, M., Cusi, K., Mandarino, S.J., Finlayson, J., et al. (2005). Lipid infusion induces an inflammatory/fibrotic response and decreases expression of nuclear encoded mitochondrial genes in human skeletal muscle. *J Biol Chem, 280*(11), 10290–10297.

Saltiel, A., & Kahn, C. (2001). Insulin signalling and the regulation of glucose and lipid metabolism. *Nature, 414*(6865), 799-806.

Sampath, H., Miyazaki, M., Dobrzyn, A., & Ntambi, J.M. (2007). Stearoyl-CoA desaturase-1 mediates the pro-lipogenic effects of dietary saturated fat. *J Biol Chem, 282*(4), 2483-2493.

Shah, O.J., Anthony, J.C., Kimball, S.R., & Jefferson, L.S. (2000). 4E-BP1 and S6K1: translational integration sites for nutritional and hormonal information in muscle. *Am J Physiol Endocrinol Metab, 279,* E715–E729.

Shimabukuro, M., Higa, M., Zhou, Y.T., Wang, M.Y., Newgard, C.B., & Unger, R.H. (1998). Lipoapoptosis in beta-cells of obese prediabetic fa/fa rats. Role of serine palmitoyltransferase overexpression. *J Biol Chem, 273*(49), 32487-32490.

Solans, R., Motta, C., Sola, R., Laville, A.E., Lima, J., Simeon, P., et al. (2000). Abnormalities of erythrocyte membrane fluidity, lipid composition, and lipid peroxidation in systemic sclerosis: evidence of free radical-mediated injury. *Arthritis Rheum, 43*(4), 894-900.

Stefan, N., Peter, A., Cegan, A., Staiger, H., Machann, J., Schick, F., et al. (2008). Low hepatic stearoyl-CoA desaturase 1 activity is associated with fatty liver and insulin resistance in obese humans. *Diabetologia, 51,* 648-656.

Storlein, L.H., Jenkins, A.B., Chisholm, D.J., Pascoe, W.S., Khouri, S. & Kraegen, E.W. (1991). Influence of dietary fat composition on development of insulin resistance in rats. Relationship to muscle triglyceride and omega-3 fatty acids in muscle phospholipid. *Diabetes, 40*(2), 280-289.

Strabble, M.S., & Ntambi, J.M. (2010). Genetic control of de novo lipogenesis : role in diet-induced obesity. *Crit Rev Biochem Micro Biol, 45*(3), 199-214.

Sumiyoshi, M., Sakanaka, M., & Kimura, Y. (2006). Chronic intake of high-fat and high-sucrose diets differentially affects glucose intolerance in mice. *J Nutr, 136*(3), 582-587.

Summers, S.A., Garza, L.A., Zhou, H., & Birnbaum, M.J. (1998). Regulation of insulin-stimulated glucose transporter GLUT4 translocation and Akt kinase activity by ceramide. *Mol Cell Biol, 18*(9), 5457-5464.

Surwit, R.S., Feinglos, M.N., Rodin, J., Sutherland, A., Petro, A.E., Opara, E.C., et al. (1995). Differential effects of fat and sucrose on the development of obesity and diabetes in the C57BL/6J and A/J mice. *Metabolism, 44*(5), 645-651.

Timlin, M.T., & Parks, E.J. (2005). Temporal pattern of de novo lipogenesis in the postprandial state in healthy men. *Am J Clin Nutr, 81*(1), 35-42.

Tremblay, F., & Marette, A. (2001). Amino acid and insulin signalling via the mTOR/p70 S6 kinase pathway. *J Biol Chem, 276,* 38052-38060.

Unger , R. H. (2003). Minireview: weapons of lean body mass destruction: the role of ectopic lipids in the metabolic syndrome. *Endocrinology, 144,* 5159-5165 .

Vodenik, B., Rovira, J., & Campistol, J.M. (2009). Mammalian target of rapamycin and diabetes: What does the current evidence tell us? *Transplant Proc*, *41*, S31-S38.

Voss, M.D., Beha, A., Tennagels, N., Tschank, G., Herling, A.W., Quint, M., et al. (2005). Gene expression profiling in skeletal muscle of Zucker diabetic fatty rats: implications for a role of stearoyl-CoA desaturase 1 in insulin resistance. *Diabetologia*, *48*(12), 2622-2630.

Wang, H. & Eckel, R.H. (2009). Lipoprotein lipase: from gene to obesity. *Am J Physiol Endocrinol Metab, 297*, E271–E288.

Wang, H., Knaub, L.A., Jensen, D.R., Jung, D.Y., Hong, E-G., Ko, H-J., et al. (2009). Skeletal muscle –specific deletion of lipoprotein lipase enhances insulin signaling in skeletal muscle but causes insulin resistance in liver and other tissues. *Diabetes, 58,* 116-124.

Warensjo, E., Ingelsson, E., Lundmark, P., Lannfelt, L., Syvanen, A-C., Vessby, B., & Riserus, U. (2007). Polymorphisms in the SCD1 gene: Associations with body fat distribution and insulin sensitivity. *Obesity*, *15*(7), 1732-1740.

Woods, A., Azzout-Marniche, D., Foretz, M., Stein, S.C., Lemarchand, P., Ferre, P., et al. (2000). Characterization of the role of AMP-activated protein kinase in the regulation of glucose-activated gene expression using constitutively active and dominant negative forms of the kinase. *Mol Cell Biol*, *20*(18), 6704-6711.

World Health Organization. (2006). *Obesity and Overweight*. Retrieved February 1, 2011, from http://www.who.int/mediacentre/factsheets/fs311/en/index.html

Zhang, L., Ge, L., Parimoo, S., Stenn, K., & Prouty, S.M. (1999). Human stearoyl-CoA desaturase: alternative transcripts generated from a single gene by usage of tandem polyadenylation sites. *J Biochem, 340,* 255-264.

Zhang, X., Qi, R., Xian, X., Yang, F., Blackstein, M., Deng, X., et al. (2008). Spontaneous atherosclerosis in aged lipoprotein lipase-deficient mice with severe hypertriglyceridemia on a normal chow diet. *Circ Res*, *102*(2), 250-256.

Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, Y., et al. (2001). Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest, 108*(8), 1167-1174.

Zhou , Y.T., Grayburn, P., Karim, A., Shimabukuor, M., Higa, M., Baetens, D., et al. (2000). Lipotoxic heart disease in obese rats: implications for human obesity. *Proc Natl Acad Sci USA, 97,* 1784–1789.

APPENDICES

Appendix 1: Animal Care Utilization Protocol Approval

UNIVERSITY OF MANITOBA | OFFICE OF RESEARCH SERVICES
Office of the Vice-President (Research)

CTC Building
208 - 194 Dafoe Road
Winnipeg, MB R3T 2N2
Fax (204) 269-7173
www.umanitoba.ca/research

8 August 2007

TO: Dr. P. Jones, RCFFN
196 Innovation Drive

FROM: Dr. T. Dick, Chair, Fort Garry Campus Protocol Management and Review Committee

RE: "Mechanisms responsible for the anti-obesity potential of stearoyl-CoA desaturase-1 inhibition using an antisense oligonucleotide: Implications of dietary fat source and energy intakes"

Please be advised that your Animal Care Utilization Protocol, reference **F07-022**, has received **approval** by the Fort Garry Campus Protocol Management & Review Committee and is valid until **31 August 2008**. The procedures described by you in the protocol have placed this research in the Category "B" of invasiveness.

It is understood that these animals will be used only as described in your protocol. The protocol must be kept current. Should changes become necessary, very minor alterations can be made with the prior written approval of a university Veterinarian and written notification of the Chair of the Fort Garry Campus Protocol Management and Review Committee. More substantive changes will require resubmission to and reassessment by the Fort Garry Protocol Management and Review Committee. If approved, this will result in the assignment of a new protocol reference number.

Failure to follow this protocol, or renew it prior to the expiry date, will result in the termination of your ability to continue using or ordering animals. The protocol reference number must be used when ordering animals.

TD:tvo

copy: Veterinary Services
Ms T. Whittington, RCFFN

Appendix 2: Animal Use Protocol Form

CONFIDENTIAL **Protocol # (for office use only)** F07-022

ANIMAL USE PROTOCOL FORM (RESEARCH/TEACHING/TESTING)

UNIVERSITY OF MANITOBA

REVISED JANUARY 25, 2007

You are strongly encouraged to read the Protocol Instructions (http://umanitoba.ca/research/ors/ethics/animal/) before completing this application. If you require assistance in completing this application, please feel free to contact Veterinary Services (Fort Garry/Bannatyne Campus @ 474-6557/474-6254/789-3469) or Dr. Randy Aitken (St. Boniface GHRC @ 235-3630).

Check one with an X: Pilot Study: (see Definition of Pilot Study in Instructions Section 1) [ ] New Application: [X] Renewal of Protocol #: [ ]

Proposed Start Date: June 1, 2007 Proposed End Date: May 31, 2008

Project Title: Mechanisms responsible for the anti-obesity potential of stearoyl-CoA desaturase-1 inhibition using an antisense oligonucleotide: Implications of dietary fat source and energy intakes.

Grant Title (if different from above):

Block 1, Principal Investigator and Emergency Contact

Principal Investigator: Dr. Peter Jones Telephone: 474-8883

Academic Rank: Professor Fax: 474-7552

Department: Richardson Centre Address: 196 Innovation Drive

E-mail: peter_jones@umanitoba.ca

Research staff to be contacted in case of animal health/endpoint concerns:

Primary: (Name) Dr. Scott Harding Office Telephone: 474-7842

After hours phone: 221-7887

Secondary: (Name) Office Telephone:

After hours phone:

Block 2, List all Personnel Involved in the Project:

List all laboratory personnel involved in the study and identify their position (investigator, student, postdoc, research associate, research technician). Schedule 1's should be filled out for each person.

UNIVERSITY OF MANITOBA
JUN 25 2007

Name	Position
Dr. Scott Harding	Research Fellow
Mr. Chris Marinangeli	PhD Candidate
Ms. Khatima Khalloufi	Research Technician

Will university animal facility staff provide any technical services beyond normal husbandry? Yes: [ ] No: [X]

If yes, please list the services provided by the university animal facility staff, i.e. blood collection, gavage, etc. Note: A Schedule 1 is not required for these individuals in this instance.

Block 3, Canadian Council on Animal Care (CCAC) Reporting Data

(Visit the CCAC web site at http://www.ccac.ca)

Protocol Description: In 40 words or less and in terms understandable to a non-scientist, provide a descriptive summary of the nature of the procedures involved in the proposed project. For example: "*Low levels of mercury exposure to fish may alter function of the thyroid gland. This study **will expose fish** to different levels of mercury and **blood samples will be collected** to assess thyroid function*". IN ADDITION, include suggested key words to describe the procedures, e.g. capture, blood collection, etc. Additional suggested key words can be found in Instructions Section 2.

Our study will investigate how such a blocker works in combination with dietary modifications and reduced energy intakes. We will also describe how inhibiting this enzyme affects other metabolic processes such as antioxidant status and protein synthesis rates.

Is this study acute or chronic? (see Instructions Section 3 for definitions): Chronic

Maximum CCAC Category of Invasiveness - A, B, C, or D (see Instructions Section 4 for explanation): B

Purpose of Animal Use – 0,1, 2, 3, 4 or 5 (see Instructions Section 5 for explanation): 2

Block 4, Animal Involvement:

Block 4A, Fill in the table below for each species you propose to use in this project.

	#1	#2	#3
Species	Mouse		
Strains	C57BL/6		

Sex	male		
Age or Weight	3 weeks		
Source	Charles Rivers		
Total # of animals requested for project duration	74		
Total # of animals requested for one year (must match total justified in Block 6, section A)	74		
Maximum number of animals to be housed at one time	32		
Estimated maximum length of time animals will be held for the duration of this project	14 weeks		

Block 4B, Animal Husbandry (place an "x" in space provided):

	Central Animal Care		St. Boniface General Hospital Research Centre
	Zoology Animal Holding Facility		Psychology Animal Holding Facility
	Animal Science Research Unit		Glenlea Research Station
	Animal Science Annex	X	RCFFN
	Animal Science Basement		Offsite Housing: Complete schedule 14
	Poultry Facility		Other (i.e. research staff) (please specify)

Wherever possible, all animal work should be conducted in designated animal holding facilities. Animals cannot be held outside designated animal facilities for longer than a 24-hour period without PMRC approval.

Are live animals ever taken to your laboratory?

Yes	No	If yes, indicate building and room #
X		RCFFN Room 2XX

Identify the procedures to be done and justify why these procedures can not be done in the animal holding facility.
Dual emission x-ray absorptiometry scan for body composition. Non-invasive.

How long do the animals remain alive in the laboratory?
7 minutes

Do the animals require a return to housing after treatment in the laboratory?
Yes (baseline scan and mid-point of feeding trial) – Last scan is post-euthanasia.

Block 4C, Transportation of Animals - Animals will be transported to the facility by: (place an "x" in space provided):

	Central Animal Care Services	X	Established Commercial Carrier

☐ Glenlea Staff ☐ Other (please specify) ☐

Block 4D, Renewal (If this is a full form renewal, answer the following. If not, proceed to Block 4).

Describe any problems that resulted in mortality or termination of animals prior to the end point of your procedures. Were these anticipated or not? Describe any changes to procedures being made to prevent recurrences.
If no such problems arose, enter "NONE".

Describe Problem:

Was this anticipated? If not, why not?:

Cause (if known):

Describe changes, to prevent recurrence:

Did **any other problems** with animals occur **as a result of procedures conducted** under this protocol? If so, list them below, give causes and state what changes will be made for this renewal to prevent them.
If no problems arose, enter "NONE".

Describe Problem:

Cause (if known):

Describe changes to prevent recurrence:

Block 5, Lay Description of the Project

Answer each of the following sections in terms **UNDERSTANDABLE TO A NON-SCIENTIST**.

Block 5A, Rationale: List the overall aims of the study and the potential benefits to human/animal health or to the advancement of scientific knowledge. See example in Instructions section 6.

Obesity and its related morbidities are a growing health concern for Canadian society. Research and development of new pharmaceutical intervention to treat obesity are a major focus for both public and private research organizations. In partnership with Merck Frosst & Company Canada we are proposing to study a novel fat synthesis blocker. Inhibiting this process, through disruption of the normal function of specific enzyme has been shown to reduce fat deposition and increase energy expenditure, primarily through increase burning of fat for energy.

Block 5B, Specific Objectives of the Study: List the objectives of this study. See example in Instructions section 6.

The proposed study will determine the effect of dietary lipid (highly saturated vs highly unsaturated) and energy intakes on the effectiveness of an inhibitory ASO of the mouse SCD-1 to increase energy expenditure and fatty acid oxidation while reducing endogenous lipogenesis. Secondary outcomes of this study are to explore the expression of key genes related to fatty acid and glucose metabolic pathways. Finally, we will also investigate the effect SCD inhibition has on both protein synthesis and oxidative stress.

The primary outcomes of the proposed project:

1. To establish if various dietary fat compositions over a 10-week period impact the known metabolic changes associated with SCD inhibition *(metabolic changes: ↑ β-oxidation, ↑ energy expenditure and ↓ adiposity, ↓ desaturation index, ↓ fatty acid and sterol synthesis).*

2. To establish if different energy intakes (100% & 70%) over a 10-week period impact the known metabolic changes associated with SCD inhibition.

The secondary outcomes of this proposed project are:
1. To establish if SCD inhibition across various dietary fat composition and energy intakes change muscle and tissue protein synthesis rates.
2. To establish if there are variations in tissue glutathione concentrations and synthesis rates.

To establish how SCD inhibition over a 10-week period affects gene expression for specific proteins in the fatty acid synthesis and catabolism pathways, glucose metabolic pathways, protein synthesis pathways and glutathione synthesis pathways.

Block 5C, Summary of the Procedures: For each experimental group, describe all procedures and techniques (including timing and duration) which will be performed on live animals. If a procedure is covered by an University of Manitoba accepted SOP, indicate the SOP # and no further detail is required. Describe how the procedures relate to the objectives of the study. See example in Instructions Section 6. If good quality pictures, diagrams, or drawings would be helpful, please feel free to submit them. If multiple procedures or treatments over some period of time (days, weeks) are part of the experiment design, you are encouraged to include a schematic time line to describe this.

Experimental Design and Analytical Methods

Animals and Study Design

The study will be conducted in 2 phases – Phase 1: C57BL/6 mice (32 per phase) will be purchased from Charles Rivers Laboratories (Montreal, QC), housed individually in hanging mouse cages at the RCFFN animal care centre (12-hour light-dark cycle) and given access (ad libitum) to study diet for 2 weeks prior to starting the study. Mice will then be randomized to a specific dietary lipid source as described in Table 1. After acclimatization, the mice will begin receiving either SCD inhibitor (15 mg/kg in 0.9% saline) or sham (0.9% saline only) i.p. injects twice weekly for 10 weeks, previously shown to significantly inhibit SCD-1 action. Food intake and body weight will be measured weekly. The 70% dietary restricted phase of the study (Phase 2) will begin after completion of the 100% phase in order to accurately account of food intakes to then set the 70% level of energy intake.

At baseline, week-5 and week-10 energy expenditure will be measured by indirect calorimetry and body composition will be measured by DEXA (while under mild anesthesia – ketamine/xylazine). The rate of fatty acid oxidation will also be measured isotopically using [1-^{13}C]palmitate, given orally, and taking a baseline breath sample followed by sampling every hour for 4-hours. Endogenous fatty acid, protein and glutathione synthesis will be measures on the following day using deuterium, L-[ring-$^{2}H_5$]phenylalanine and U-[^{15}N, $^{13}C_2$]glycine incorporation, respectively. L-[ring-$^{2}H_5$]phenylalanine and U-[^{15}N, $^{13}C_2$]glycine (150 µmol/100g BW; 40 moles %) in deuterated water (D_2O, 99 moles %) will be injected i.p. on the last day of the study. After 60 minutes post injection each mouse will be anesthetized using isoflurane and blood sampled via cardiac puncture. Following blood sampling the mice will be euthanized by an overdose of sodium pentobarbital and tissues sampled (liver, heart, hindlimb, l. dorsi and adipose). Tissues will be rinsed in ice-cold saline, partitioned and frozen immediately by immersion in liquid N_2.

Diet

The diet is outlined in Table 2 and at the 100% level supplies the required macro and micronutrients for the animals. The energy reduced adjusted diet allows for the same total intake of fat while reducing carbohydrate. By feeding this diet at 70% of the dietary intake of the complete diet each animal will receive 100% protein, fat and vitamin/mineral requirements but reduced carbohydrate levels to allow for equivalent intakes of total fat despite reduced energy intake.

List of Procedures

Intraperitoneal injection - Xylazine–Ketamine 0.1ml/10g (for DEXA scan)
Intraperitoneal injection – antisense oligonucleotide inhibitor of SCD-1
Energy expenditure – non-invasive by indirect calorimetry
Intraperitoneal injection – deuterated water with amino acid tracers (sterile solution and stable isotopes)
Oral gavage – stable isotope labeled fatty acid.
Anesthesia – inhaled isoflurane using induction chamber
Cardiac puncture blood terminal blood sampling
Euthanasia – overdose of sodium pentobarbital (110mg/kg)

Block 6, Justification of Animal Usage and the 3 R's (Replacement, Reduction, and Refinement)

Answer the following sections in terms **UNDERSTANDABLE TO A NON-SCIENTIST.**

Block 6A: Justify the number of animals requested for each species as described in Block 4A, based on the experimental objectives of the project. Include information on experimental and control groups, # per group, and failure rates or justify in terms of statistical requirements, product yield, past research experience, etc. If you have multiple experiments proposed, a breakdown of the various stages of the research (which experiments with how many animals will be done when) may be included. This is particularly useful when a multi-year study is outlined but animals for one year are highlighted in the present protocol. Alternatively, use the table below and include a justification for the group size.

1. 10 mice will be required to determine the feasibility of collecting all listed analytical endpoints. These mice will also be used to determine what the optimal dose of stable isotopes to be used is and weather there is any interference with analytical endpoints of interest.
2. 16 mice (4 per dietary group) will be taken through the protocol for the purpose of establishing baseline isotope enrichment of the animals. These animals will not be subject to any of the protocols listed about except the terminal blood sampling and euthanasia.
3. The remaining 64 mice in the study design listed here is the minimum number of animals per group (n = 8) which will demonstrate a 10% statistical difference in the primary outcome of fatty acid synthesis rates at P < 0.05 and > 80% statistical power.

Test Agents or Procedures	# of Animals and Species per Group	# of Dosages and/or Routes of Administration	# of Endpoints	Other variables (i.e. age, sex, weight, etc.)	Total number of Animals
e.g. 2 drugs	**e.g. 6 rats**	**e.g. . 6 variables (0, .03, .05 mg/kg - IM, IP)**	**e.g. 3 variables (1,7, 10 days)**	**e.g. 2 variables (male, female groups)**	**2x6x6x3x2=432**
Pilot	2	5	1	1	10
Isotope control	4	2	1	2	16
2	8	2	1	2	64

Block 6B, Are there any alternatives to animal use (e.g. cell cultures, mathematical models, computer simulations) that would suffice for meeting the objectives of this project? If yes, justify below, your request to use animals.

The analytical endpoints are metabolic products of in vivo metabolic processes (fatty acid production, protein synthesis, tissue specific gene expression) which involve multiple organs. There are no current suitable alternatives.

Block 6C, Describe the characteristics of the animal species selected that justifies use in the proposed study (consider characteristics such as body size, species/strain, data from previous studies or unique anatomic/physiological/genetic features).

The test article in this study is an antisense oligonucleotide (ASO) which inhibits the action of stearoyl-CoA desaturase-1 (EC 1.14.19.1). The C57BL/6 mouse was chosen for this study because of its small size (lower quantity of ASO required) and previous used in a similar study (J. Clin. Invest. 115: 1030 (2005)).

Block 6D, To reduce animal use, would your animals/ tissue be suitable for transfer to another project at the conclusion of your experiments?

We require the use of the following tissues in the animal; blood samples, heart, liver, and skeletal muscle.

Block 6E, What provisions will be made to provide environmental enrichment for animals used in this study? If an approved facility SOP or practice on environmental enrichment is available, indicate the SOP # and no

further detail is needed. If any restrictions (beyond normal husbandry practices) are required regarding environmental enrichment (i.e. no group housing), please identify them and provide scientific justification.
If no environmental enrichment is being provided, this must be justified.

There will not be groups housing as the dietary intake of each mouse needs to be recorded throughout the study. The energy intake for 32 mice will be restricted by 30% of the mice receiving full feeds.

Block 7, Euthanasia/Adoption/Disposition of Animals and Humane Endpoints

Block 7A, Is euthanasia a requirement of this study?

If yes, describe the method. If chemical agents are being used, include the dose and route. **Note:** A secondary physical method (e.g. removal of heart, exsanguination, cervical dislocation, double pneumothorax) is required following chemical euthanasia. Indicate what secondary method will be utilized.

Yes, blood sampling will require cardiac puncture followed by euthanasia by overdose of sodium pentobarbital (110mg/kg). Several tissues (liver, heart, skeletal muscle) will then be harvested.

If euthanasia is not required, indicate what will happen to the animals at the end of the experiment.
Where possible, adoption of suitable animals is strongly encouraged.

- ☐ Adoption
- ☐ Transfer to other project(s)
- ☐ Other (please specify)
- ☐ Return to herd
- ☐ Commercial purpose

Block 7B, If a physical method of euthanasia is to be used **without chemical agents**, provide a scientific justification.

No physical euthanasia is required.

Block 7C, If this protocol is an "A" or "B" category of invasiveness and early endpoints are applicable, list them below. If this protocol is a "C" or "D" category of invasiveness, Schedule 4, "Humane Endpoints" must be completed.

Block 7D: Should an animal become ill during your study, are there medical interventions that would be contraindicated? If yes, please describe and justify.

No, however the animals would become useless to the endpoints analysis as any sort of infection or inflammation could alter the feed intake and other primary endpoints.

Block 8, Funding

Agency/Source: Merck Frosst Canada and NSERC

Awarded: February 2007 (Merck Frosst) Applied for: April 2007 (NSERC)

Funding Start Date: April 2007 Funding End Date:

Does this source conduct peer review? (If no, see Instructions Section 7 for obtaining peer review).	Yes
Was the project described in this protocol included in the proposal that was approved for funding? (If not, see Instructions Section 7 for obtaining peer review).	Yes: X No:

Block 9, Schedules

Check yes or no for all sections below and complete the required schedule for each section checked yes.

	Yes	No
Schedule 1A and 1B, Documentation (Must be completed by the principal investigator (1A) and all research personnel (1B).	X	
Schedule 2, Anesthesia/Chemical Restraint (When anesthetic/chemical restraint agents are being used).	X	
Schedule 3, Surgery (When surgical procedures are being performed).		
Schedule 4, Humane Endpoints (Required for C or D category of invasiveness experiments. The schedule will ask you to provide a description of conditions that may cause distress/discomfort, how they will be identified and what will be done to alleviate them).		
Schedule 5, Restraint (For any restraint longer or more severe than normally required for examination, injection or a single blood collection in conscious animals, or restricted housing, e.g. metabolism crates/cages).	X	
Schedule 6, Feed/Water/Nutrient Alteration (For any period of fasting (including water), except pre-surgical, any force feeding or any alteration to the diet in which (a) NRC requirements are not being met; (b) feedstuffs not normally fed are being used; (c) changes to the diet are being made to produce physiological changes in the animal).	X	
Schedule 7, Behavioural Experiments (If the project involves behavioural manipulation, shock, negative reinforcement, predator/prey relationships, or sensory deprivation).		
Schedule 8, Environmental Manipulation (If the project involves environmental manipulation or imposes any potential adverse environmental effect).		
Schedule 9, Teaching (When the main purpose of animal use is education, including courses, workshops, demonstrations, etc.)		
Schedule 10, Risk Assessment (**To be completed if any of the administered agents of the protocol meet the criteria described on the schedule cover page).**		
Schedule 11, Field Studies (For field studies and when the project involves capture or release of animals into the wild).		
Schedule 12, Common Procedures (To provide more detail for common procedures including blood and/or tissue collection (including tail snips and ear punches); individual marking; injections; catheter placement; gavage; indwelling osmotic pumps.) Injectable anesthetic and euthanasia agents need not be listed here.	X	
Schedule 13, Genetically Altered Animals (If using transgenic, knockout, knock-in or mutant animal).		
Schedule 14, Offsite Housing (If the project involves the use of animals on non-university property, excluding SBGHRC and CancerCare Manitoba).		
Schedule 15, Establishment and Maintenance of Breeding Colonies (If breeding of any species occurs, regardless if for maintenance of species or for research/teaching purposes).		

Block 10, Declaration

The signature of the principal investigator below confirms that the information in this application is exact and complete. It assures that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and the University of Manitoba Policy 1404, Care and Use of Animals. It also assures that prior to any deviations from this protocol being performed, the applicable veterinarian and PMRC chair must approve an amendment. Substantial changes will require re-submission to the PMRC. It is understood that this approval is valid for one year and that protocols must be approved on an annual basis prior to the expiry date.

Principal Investigator: [redacted] Date: June 15, 07

Chair, Protocol Management and Review Committee: [redacted] Date: Aug 8/07

January 25, 2007

Schedule 2, Anesthesia/Chemical Restraint

(please complete a separate schedule for each procedure involving different agents or purposes)

1. **Why is the animal being anesthetized or chemically restrained?**

Chemical restraint for DEXA scanning; anesthesia prior to terminal blood draw and euthanasia.

2. **Premedications/Chemical Restraint Agents** (Sedatives/Tranquilizers/Anticholinergics/Analgesics)

Drug	Dose (mg/Kg)	Administration Route	Duration of Action for Chemical Restraint

3. **Injectable Agents for Induction or Maintenance**

Induction

Drug	Dose (mg/Kg)	Administration Route
Xylazine–Ketamine	10/100 mg per Kg (respectively)	IP

Maintenance

Drug	Dose/Infusion Rate	Administration Route	Top-up Dose

4. **Inhalent Anesthetic Agents for Induction or Maintenance**

a) **Induction**

Drug	Concentration/%	Method (ie chamber/mask)
Isoflurane	5%	Chamber

b) **Maintenance**

Drug	Concentration/%	Are you intubating?
Isoflurane	2%	Mask no intubation

c) If intubation is to be performed, describe the procedure and identify the supplies to be used (e.g. lidocaine spray).

5. **Estimated Duration of Anesthesia Requirements:**

< 15 minutes for each.

6. **Method(s) of Monitoring Anesthetic Depth:** e.g. pedal reflex for neuromuscular system, ECG for cardiac system, respiratory rate, blood pressure EEG, heart rate, respiratory rate, etc.

pedal reflex for neuromuscular system

7. **Other Anesthetic Agents/Supplements: eg, local anesthetics, reversing agents**

Drug	Dose (mg/Kg)	Administration Route

8. **Post-Anesthetic Care Plan: Provide a detailed summary of how the animals will be monitored in the post-anesthetic period if surgery IS NOT being performed. If surgery is being performed, include Schedule 3, "Surgery".**

January 25, 2007

9. Neuromuscular Blocker (NMB)

NMB: **Dose (mg/Kg):**
Administration Route: **Duration of Action:**

9a. At what point in the procedures will the NMB be administered?

9b. Methods of monitoring anesthetic depth once the NMB has been administered.

9c. Provide evidence that the monitored parameters are appropriate for this species.

9d. Scientifically justify the use of a NMB. What phases of this protocol could not be done without the use of NMBs and why? Is the use of NMBs limited to these phases?

9e. Has the anesthetic regimen been shown to achieve a surgical plane of anesthesia in the absence of the NMB in this species?
If not, a pilot study must be performed to determine if the anesthetic regimen is appropriate.

9f. Indicate how you have chosen your NMB in order to utilize the lowest dose or for the shortest period of time.

9g. Describe the experience and qualifications of the person using the NMB (identified below), including experience with this anesthetic regime in this species. This person must be present at all times when a NMB is in use.

Name and signature of person (described above) using the NMB. This person must be present at all times when a NMB is in use.

Name: Signature: Date:

Name and signature of principal investigator FOR PROTOCOLS USING NMBs ONLY.

Name: Signature: Date:

Reviewed prior to submission by Clinical Veterinarian
Signature of Veterinarian: Date:

Final Review by Clinical Veterinarian
Signature of Veterinarian: [redacted] Date: June 28/07

January 25, 2007

Schedule 5, Restraint

1. Why is restraint necessary?

   For respiratory gas analysis

2. Describe the type of restraint, including the equipment, etc. to be used. What negative effects are possible? If drugs are used, complete Schedule 2.

   The mice will be placed in individual chambers for 2 hours while their respiratory gas is measured. There should be no negative effects from this procedure as none have been observed in the past.

3. Describe the preconditioning procedures that will be used to help adapt the animals. If none can be used, indicate why.

4. Describe the periods of restraint (e.g. for 12 h once a week for 3 weeks).

   One time for 2 hours on the day of euthanasia.

5. Location (building and room #) where restraint will occur.

   RCFFN , animal holding facility, room 137B

6. For housing restraint, indicate the size of the animal and the size of the restraint device. For growing animals, indicate how the restraint will be modified to account for growth or indicate the maximum size of the animal if the restraint will not be modified.

   The mice will be placed in a container appropriate in size to allow for full movement and exploration

7. What criteria will be used for early termination of the restraint?

   n/a

8. What normal activities of the animal, such as ability to stretch, lie comfortably, turn around, groom, locomote, feed, drink, etc. are restricted or eliminated while restrained?

   The mice will be deprived of feed and water during this time

9. What other techniques have you considered to avoid or reduce the level and duration of restraint? (For example: telemetry, sedation). Why have you chosen not to use them?

Note: Additional justification is required for restraint beyond the following guidelines:

Restraint by any method which does not allow movement of the limbs or neck for $\geq$ 1 hour in unconditioned animals or $\geq$ 2 hours in conditioned animals.

Restraint which does not allow normal stretching or rearing behaviour, resting in lateral recumbency or the ability to turn around for $\geq$ 6 hours in unconditioned animals for $\geq$ 10 hours in conditioned animals. In these situations, it will be necessary to release the animal from restraint for 1 hour of exercise at the end of 6 hours or 10 hours before resuming restraint. (For those species used in agriculture research where specific types of restrictive housing are accepted practice, e.g. gestation stall for swine, the

Codes of Practice are accepted).

Restraint in housing smaller than the normal area, such as large animal metabolism crates. (CCAC Guidelines: Restraint should not exceed 21 days and may not exceed 30 days. Animals must be released from crates for at least 3 hours every 7 days).

January 25, 2007

Schedule 6, Feed/Water/Nutrient Alteration

1. Nutrient Deprivation: All fasting other than for surgery/anesthesia (which is included in the surgery/anesthesia schedule), should be indicated. For example, fasted blood samples, time in rodent metabolism cages without feed, etc.

1a. Complete the following table.

Category	Duration of Deprivation	% of NRC Requirement or *ad lib.* Consumption
All feed	10 weeks	70% of ad lib consumption
All water	N/A	N/A
Partial feed	N/A	
Partial water	n/A	

1b. If specific nutrient(s) are withheld, please list individually below.

Nutrient Withheld	Duration of Deprivation	% of NRC Requirement or *ad lib.* Consumption

1c. What is the rationale for the proposed level of deprivation?

Providing 70% of ad lib consumption is meant to simulate a mild caloric restricted diet based on ad lib consumption from the 100% diet groups. Inhibition of the enzyme (SCD-1) may not be as affective in a food restriction model as in an ad lib dietary situation.

1d. For the specific nutrients named in the above table, give the ranges of signs of deprivation, which are to be expected given the degree and duration of deprivation. These signs must be taken into consideration when determining early endpoints (see Block 6C).

2. Nutrient Supplementation: The addition of nutrients, excluding drugs, must be indicated e.g. force feeding, cholesterol additions to diet, etc.

2a. Complete the following table.

Force-feeding	Quantity to be Utilised	% of NRC Requirement or *ad lib.* Consumption
- Normal Diet		
- Water Intake		

2b. Indicate how the force-feeding will be accomplished and with what technique including equipment to be used.

2c. For supplementation with specific nutrients, complete the following table.

Specific Nutrient	Quantity to be Utilised	% of NRC Requirement or *ad lib.* Consumption

2d. What is the rationale for the proposed addition to the diet?

2e. For the specific nutrients named in the above table, please provide the range of signs expected given the additions to the diet. These signs must be taken into consideration when determining early endpoints (see Block 6C).

3. Nutrient/Feedstuff Substitution (for unusual/new feedstuff testing)

3a. Within the diet, what dietary items are being introduced and removed?

3b. Does substitution affect the nutritional balance? If yes, how?

3c. Is the new diet being tested for adverse effects? If so, which ones? If not, are any adverse effects anticipated?

4. Non-approved, Non-registered Feeds

Will the proposed project involve feeding livestock feeds that are not currently approved or registered in Canada (novel or imported feed ingredients)? Yes_____ No_____

If "yes", you must contact the "Feed Section" of the Canadian Food Inspection Agency for an "Authorization of the Release of Novel Feeds for Research Purposes". For more information please contact:

Feed Section
Animal Health and Production Division
Canadian Food Inspection Agency
59 Camelot Drive
Ottawa, ON, Canada K1A 0Y9
Ph. (613)-225-2342
Fax. (613)-228-6614
Website: http://www.inspection.gc.ca/english/anima/feebet/feebete.shtml

It is the responsibility of the Primary Investigator to arrange for permits from CFIA to cover disposal of feeds and/or animals when novel or imported feed sources are utilized (contact the above address or website for a list of approved ingredients).

5. Non-nutrient Feed/Water Additives (test compounds/medications)

	Substance 1	Substance 2	Substance 3
Substance			
Concentration			
Route			
Timing			

5a). Will the addition of the test substances cause concerns with palatability and/or feed/water refusal?

5b). Will the addition of the test substances interfere with the release or availability of other nutrients?

January 25, 2007

Schedule 12, Common Procedures

(includes blood and/or tissue collection (including tail snips and ear punches); individual marking; injections; catheters; gavage; indwelling osmotic pumps)

1. Blood Collection

1a. If blood is to be collected, the table below MUST be completed. If more than 3 quantities will be withdrawn, attach a second Schedule 12.

	Collection 1	Collection 2	Collection 3
Volume (ml)	Maximum		
Timing of collections e.g. 0 h, 1 h, 2 h, and 14 d.	Terminal		
Animal Weight Range (g or Kg)	30-40 g		

1b. Describe method(s) of blood collection including vein, needle size, and procedure or indicate SOP #.

After being anesthetized isoflurane the mice will be exsanguinated via cardiac puncture. A 25g 5/8" needle will be used with a 1cc syringe. The needle will be inserted at a 30^0 angle, just to the left of the xyphoid process and caudal to the bifurcation of the ribs. Immediately after sample collection the animal will be euthanized by overdose of sodium pentobarbital (110mg/kg).

2. Tissue Collection (including ear punch and tail snips for genotyping).

2a. Will tissues be collected? **If YES**, what tissues will be collected and how?

Yes, Liver, heart, hindlimb muscle, L. dorsi and adipose tissue

2b. Will tissues be collected only after euthanasia? **If NO**, provide details of tissue collection, including method, time of collection, method of restraint, etc.

Yes

3. Indwelling Catheters

Will in-dwelling catheters (venous, arterial, urinary) be used? If yes, provide details, including duration, insertion and maintenance of the catheter. **Note: If surgery or anesthesia is required, complete and attach Schedules 2 and/or 3.**

No

4. Transponders or Transmitters

4a. Will transponders or transmitters be implanted? **Note: If surgery or anesthesia is required, complete and attach Schedules 2 and/or 3.**

No

4b. If non-surgical implantation is used, provide the size and type of implant, implanting procedure, duration of implant, and method of removal (if applicable).

No

5. Animal Marking

Will animals be individually marked for this study? If YES, describe the marking technique, any potential adverse effects of marking on the animals, and what will be done to minimize those effects.

Yes, ear punch performed by Teri Whittington – Animal Care Technician.

6. Animal Injections

If animals are being injected, the first table immediately below must be completed. If the substance was not purchased commercially, the second table immediately below must also be completed. **Note:** All agents being injected must be listed, including contents of control or sham injections. Injectable anesthetic and euthanasia agents need not be listed here.

Space is provided for four (4) different commercially available substances. If more space is required, attach a second Schedule 12.

	Substance 1	Substance 2	Substance 3	Substance 4
Substance	Antisense oligonucleotide	Deuterated water with stable labeled phenylalanine and glycine		
Volume	0.1 ml	0.2ml		
Route	IP	IP		
Timing (e.g. 3h, 4d, 10d into trial)	2x weekly	2 hours before euthanasia on final day.		

Space is provided for four (4) different non-commercially available substances. If more space is required, attach a second Schedule 12.

	Substance 1	Substance 2	Substance 3	Substance 4
PH	Neutral - PBS	6.5 – D_2O		
Sterilization Method	Sterile membrane filtered (0.22 µm) solution	Membrane filtered solution		

7. Gavage

If gavage or some other method of force feeding will be used, complete the table below.

	Substance 1	Substance 2	Substance 3	Substance 4
Substance	13C-labeled palmitic acid in vegatable oil			
Volume	0.1 ml			
Timing	Three days prior to euthanasia			

Describe method(s) of gavage including equipment used and procedure or SOP#.

Standard disposable 1 ml BD syringe with flexile plastic mouse gavage tips. Before beginning the gavage tube will be measured externally from the tip of the mouse's nose to the last rib so it is known how much of the tube can be passed into the mouse with out damaging the stomach. Restraining the mouse in a lateral position the tube will be passed into the mouth on either the left or right side of the mouth. From here the tube will be passed through the esophagus and into the stomach. If the mouse is struggling excessively or there is resistance to the passage of the tube then the tube will be removed and the procedure will be attempted again.

8. Osmotic Pumps

If indwelling osmotic pumps are to be used, complete the table below.

Species	Weight Of Animal	Pump Manufacturer	Model #	Days from Date of Implantation till endpoint	# of days the pumps may remain implanted before the Manufacturer Recommends Removal

If the pump is to remain in place for longer than the manufacturer's recommended pumping time, the possibility of leakage of concentrated saline from the pump does exist. This can result in irritation of surrounding tissues and exposure of the pump in some cases.

If the number of days from date of pump implantation to animal endpoint date exceeds the recommended time from the manufacture, please indicate what steps will be taken to deal with concerns that may arise from extended pump implantation?
(background information: http://www.alzet.com/products/imp_exp.php)

Appendix 3: Application for Amendment to Animal Use Protocol

UNIVERSITY OF MANITOBA

UNIVERSITY OF MANITOBA
FEB 27 2008
RESEARCH SERVICES & PROGRAMS

January 25, 2007

APPLICATION FOR AMENDMENT TO ANIMAL USE PROTOCOL

NOTE: An amendment may be used for minor changes in numbers of animals, changes, additions or deletions of species being used and minor modifications to procedures. Substantial changes to procedures or addition of or change to new procedures not reviewed in the existing protocol, or large changes in numbers or species of animals being used will require submission of a new protocol.

Protocol Number: F07-022 Expiry Date: August 31 2008

Principal Investigator: Peter Jones Telephone: 474-8883

Academic Rank: Professor Fax: 474-7552

Department: Richardson Centre Address: 196 Innovation Drive

E-mail: Peter_jones@umanitoba.ca

Nature of Amendment	
Change in Personnel and/or Supervision Level	
Addition or Change in Species	
Change in Animal Numbers	
Change in Procedures	X
Other	

1. Change in Personnel and/or Supervision Level (check applicable box and complete as indicated)

	Change in Staff		
Name		Addition (submit a Schedule 1)	Deletion

	Change in Supervision Level (see below).			
Name		Procedure	Supervision Level	Person to provide supervision (if applicable)
			( ) direct ( ) indirect ( ) supervisor	
			( ) direct ( ) indirect ( ) supervisor	
			( ) direct ( ) indirect ( ) supervisor	
			( ) direct ( ) indirect ( ) supervisor	

UNIVERSITY OF MANITOBA
FEB 15 2008
RESEARCH SERVICES & PROGRAMS

Supporting Documentation of Competency – **To establish your competency as it relates to the procedures identified above, please indicate the following:**

1. The number of times you have performed the procedure, the nature and rate of complications, last time performed and how/when/where the skill was acquired;
2. Assessment of competency. This should be completed by the principal investigator or identified designate using a scale of 1-5 with 1 being poor and 5 being excellent;
3. Reference to publications in which these procedures/methods are described.

2. Removal or Addition of Species/Strains/Lines

Please complete the following table.

Species or Strains Currently Approved	Number Approved	Species/Strains to be Removed From Protocol	Species/Strains to be Added To Protocol	Number Requested Total	Max Time

Justify the need for the new species, strain, or line.

NOTE: If genetically altered animals are to be used, Schedule 13 (Genetically Altered Animals) **MUST** be completed. If Schedule 13 was submitted with the original protocol, please specify below any amendments to it which these changes necessitate.

3. Change of Numbers of Animals Required. Include numbers for any species/strains/lines identified in section 2.

Species or Strain	Number Approved in Protocol	Additional # Being Requested	Total	Max. Any Time

Justification for the change (for example, in terms of statistical requirements, required product yield etc.)

4. Changes in Anesthetic and/or Analgesic Drugs Presently Approved For Administration to Animals

For changes in anesthetic and/or analgesic drugs, complete the following table.

Anesthetic/Analgesic Now Approved	Dose	Route	To be Replaced by	Dose	Route	Additional Anesthetics/ Analgesics Requested	Dose	Route

Reason for the changes.

5. Changes to Drugs or Other Compounds Given to Animals

Please complete the following table.

Drugs or Compounds Now Approved	Dose	Route	To be Replaced by	Dose	Route
Antisense oligonuceotide	15 mpk	IP	Compound-A (Merck Frosst – proprietary)	3 – 5 mpk	Oral gavage, 0.5% methocellulose suspension as vehicle.

Please specify any expected side effects that may result from each of these changes.

Compound A is not a hazardous material. Global inhibition of SCD-1 on a long term, chronic basis will result in alopecia, dermatitis and reductions in the production of eye lubrication. Acute effects are limited to impaired endogenous production of monounsaturated fatty acids. The revised compound, dose and duration will have no side effects on the animals' health and/or well being.

Reason for the changes.

The ASO has been replaced by compound-A because of the reported alopecia, dermatitis and eye lubrication issues that result from long term inhibition of SCD-1. Initially the ASO was the most cost effective alternative to the long term effect however shifting to a shorter duration of SCD-1 inhibition now makes the current Merck Frosst proprietary compound the most reasonable choice for this experimental model. The study will now look at a 3 day knockdown of the enzyme's activity at the end of the pre-conditioning phase for diet induced obesity.

Note: If any of the changes above involve hazardous drugs or agents, Schedule 10 (Potential Hazards) must be completed and submitted.

6. Minor Procedural Changes

If major procedural changes are to be made in this project, a new protocol must be submitted. Consult the Clinical Veterinarians or the Chair of your LAUC for help in deciding whether your changes are minor or major.

Specify below any minor procedural changes and the reasons for making them.

The twice weekly IP injections of the inhibitor (ASO) have been removed from the protocol replaced by 3 daily oral gavages on the final 3 days of the protocol. IP injection of the stable isotope tracers on the final day of the protocol remain unchanged.

The stable isotope tracer phenylalanine has been removed from the protocol as the endogenous deuterium labeling of alanine by the administered D_2O can be used for the protein synthesis measures – leaving only U-glycine in D_2O/0.45% saline.

Minor changes to the diet have been made – peer review of the protocol by NSERC pointed out some technical issues with using a diet high in coconut oil and recent advances in the published literature, since the time of the initial submission, demonstrate that oleate content of the diet maybe the critical point controlling the SCD-1 metabolic effects. Furthermore, the metabolic response to SCD-1 inhibition in diet induced obesity from high fat diets versus high carbohydrate diets differs. Changes to the diet are outlined in Table 1 of the attachments.

Baseline and week 5 saphenous vein blood sample (10 µl) are being added for the measurement of blood glucose concentrations during preconditioning.

* See attached pages revised block 5c
Schedule 12 revised

7. Change in Funding and/or Title of Project

Please list any changes in funding source(s).

The project is now fully funded by Merck Frosst and NSERC under the NSERC CRD # 349679-06. The end date has now changed to December 2009 – could the animal care protocol be amended to reflect this change?

Does this source conduct peer review? (If no, see Instructions Section 7 for obtaining peer review). YES

Was the project presently under review included in the proposal that was approved for funding? (If not, see Instructions section 7 for obtaining peer review). Yes: X No:

If any of the above changes make a change in the project title appropriate, please give the revised project title.

NO

If the project title is different than the grant title, please indicate the grant title.

NO

Declaration

The signature of the principal investigator below indicates agreement to all terms and conditions applied to the original protocol and this amendment. No other changes can be made to this protocol without further approved amendments or submission and approval of a new protocol to cover them.

Principal Investigator: [redacted] Date: 2008-02-12

Protocol Approved By:

Chair, Protocol Management and Review Committee: [redacted] Date: Feb 25/08

Clinical Veterinarian: [redacted] Date: Jan 22/08

January 25, 2007

Harlan Teklad — *Custom Research Diets*

TD.07910 2% Corn Oil Diet (O)

Formula	g/Kg
Casein, "Vitamin-Free" Test	190.0
DL-Methionine	3.0
Sucrose	486.6
Corn Starch	41.06
Maltodextrin	150.0
Corn Oil	20.0
Cellulose	50.0
Mineral Mix, AIN-93G-MX (94046)	35.0
Potassium Phosphate, monobasic	8.79
Calcium Carbonate	3.75
Magnesium Oxide	1.35
Ferric Citrate	0.29
Vitamin Mix, Teklad (40060)	10.0
TBHQ, antioxidant	0.01
Orange Food Color	0.15

Footnote

A high sucrose diet modified from TD.03045 to double the fat from 1-2% (2.5 - 5% of kcal from fat). Fat added at the expense of casein.

Selected Nutrient Information [1]

	% by weight	% kcal from
Protein	17.4	19.4
Carbohydrate	67.8	75.6
Fat	2.0	5.0
Kcal/g 3.6		

[1] Values are calculated from ingredient analysis or manufacturer data

Speak With A Nutritionist

- 800·483·5523
- askanutritionist@teklad.com

Key Features

- Purified Diet
- High Sucrose
- Low Fat

Key Planning Information

- *Products are made fresh to order*
- Store product at 4°C or lower
- Recommended use within 6 months
- Box labeled with product name, manufacturing date, and lot number
- Lead time:
  - 2 weeks non-irradiated
  - 4 weeks irradiated

Product Specific Information

- 1/2" Pellet or Powder (free flowing)
- Minimum order 3 Kg
- Irradiation available upon request

Options (Fees Will Apply)

- Rush order (pending availability)
- Irradiation (see Product Specific Information)
- Vacuum packaging (0.5, 1, 2, 2.5 Kg)

International Inquiry

Outside U.S.A. or Canada

- askanutritionist@teklad.com

Place Your Order (U.S.A. & Canada)

Place Order · Obtain Pricing
Check Order Status

- 800·483·5523
- 608·277·2066 *facsimile*
- customerservice@teklad.com

January 25, 2007

Schedule 12, Common Procedures

(includes blood and/or tissue collection (including tail snips and ear punches); individual marking; injections; catheters; gavage; indwelling osmotic pumps)

1. Blood Collection

1a. If blood is to be collected, the table below MUST be completed. If more than 3 quantities will be withdrawn, attach a second Schedule 12.

	Collection 1	Collection 2	Collection 3
Volume (ml)	10 µl	10 µl	Maximum
Timing of collections e.g. 0 h, 1 h, 2 h, and 14 d.	0 weeks baseline	5 weeks	Terminal
Animal Weight Range (g or Kg)	30-40 g	30-40 g	30-40 g

1b. Describe method(s) of blood collection including vein, needle size, and procedure or indicate SOP #.

Collections 1 and 2 will be collected via standard saphenous vein puncture with a hypodermic needle and collection of the drop.
After being anesthetized isoflurane the mice will be exsanguinated via cardiac puncture. A 25g 5/8" needle will be used with a 1cc syringe. The needle will be inserted at a 30^0 angle, just to the left of the xyphoid process and caudal to the bifurcation of the ribs. Immediately after sample collection the animal will be euthanized by overdose of sodium pentobarbital (110mg/kg).

2. Tissue Collection (including ear punch and tail snips for genotyping).

2a. Will tissues be collected? **If YES**, what tissues will be collected and how?

Yes, Liver, heart, hindlimb muscle, L. dorsi and adipose tissue

2b. Will tissues be collected only after euthanasia? **If NO**, provide details of tissue collection, including method, time of collection, method of restraint, etc.

Yes

3. Indwelling Catheters

Will in-dwelling catheters (venous, arterial, urinary) be used? If yes, provide details, including duration, insertion and maintenance of the catheter. **Note: If surgery or anesthesia is required, complete and attach Schedules 2 and/or 3.**

No

4. Transponders or Transmitters

4a. Will transponders or transmitters be implanted? **Note: If surgery or anesthesia is required, complete and attach Schedules 2 and/or 3.**

No

4b. If non-surgical implantation is used, provide the size and type of implant, implanting procedure, duration of

implant, and method of removal (if applicable).

No

5. Animal Marking

Will animals be individually marked for this study? If YES, describe the marking technique, any potential adverse effects of marking on the animals, and what will be done to minimize those effects.

Yes, ear punch performed by Teri Whittington – Animal Care Technician.

6. Animal Injections

If animals are being injected, the first table immediately below must be completed. If the substance was not purchased commercially, the second table immediately below must also be completed. **Note:** All agents being injected must be listed, including contents of control or sham injections. Injectable anesthetic and euthanasia agents need not be listed here.

Space is provided for four (4) different commercially available substances. If more space is required, attach a second Schedule 12.

	Substance 1	Substance 2	Substance 3	Substance 4
Substance	Deuterated water with stable labeled U-glycine			
Volume	0.2ml			
Route	IP			
Timing (e.g. 3h, 4d, 10d into trial)	2 hours before euthanasia on final day.			

Space is provided for four (4) different non-commercially available substances. If more space is required, attach a second Schedule 12.

	Substance 1	Substance 2	Substance 3	Substance 4
PH	Neutral - PBS	6.5 – D_2O		
Sterilization Method	Sterile membrane filtered (0.22 µm) solution	Membrane filtered solution		

7. Gavage

If gavage or some other method of force feeding will be used, complete the table below.

	Substance 1	Substance 2	Substance 3	Substance 4
Substance	13C-labeled palmitic acid in vegatable oil	Compound – A Proprietary Merck Frosst in 0.5% methylcellulose suspension		
Volume	0.1 ml	0.1 ml		
Timing	Three days prior to euthanasia	Final 4 days of study		

Describe method(s) of gavage including equipment used and procedure or SOP#.

Standard disposable 1 ml BD syringe with flexile plastic mouse gavage tips. Before beginning the gavage tube will be measured externally from the tip of the mouse's nose to the last rib so it is known how much of the tube can be passed into the mouse with out damaging the stomach. Restraining the mouse in a lateral position the tube will be passed into the mouth on either the left or right side of the mouth. From here the tube will be passed through the esophagus and into the stomach. If the mouse is struggling excessively or there is resistance to the passage of the tube then the tube will be removed and the procedure will be attempted again.

8. Osmotic Pumps

If indwelling osmotic pumps are to be used, complete the table below.

Species	Weight Of Animal	Pump Manufacturer	Model #	Days from Date of Implantation till endpoint	# of days the pumps may remain implanted before the Manufacturer Recommends Removal

If the pump is to remain in place for longer than the manufacturer's recommended pumping time, the possibility of leakage of concentrated saline from the pump does exist. This can result in irritation of surrounding tissues and exposure of the pump in some cases.

If the number of days from date of pump implantation to animal endpoint date exceeds the recommended time from the manufacture, please indicate what steps will be taken to deal with concerns that may arise from extended pump implantation?
(background information: http://www.alzet.com/products/imp_exp.php)

Dietary Changes:

Previous protocol proposed to use the following diet:

Table 1. Experimental Diet Composition

Ingredients	Energy Intake (% of *ad libitum*)	
	100%	70%
	g/100g diet	
Casein	22.4	32
Corn Starch	31.2	9
Sucrose	11.2	3.5
Cellulose	6.2	8.9
Vitamin Mix[a]	1.2	1.7
Mineral Mix[b]	8.6	12.3
Fat[c] (4 diets)	19	27.2
Energy[d] (kcal/100g)	418	419

[a] Harlan Teklad vitamin mix (CA. 40060, Harlan Teklad, WI, USA)

[b] AIN-93M mineral mix (TD 94049, Harlan Teklad, WI, USA)

[c] Fat mixture:

1. 100% diet-low unsaturated fatty acid content (80% SFA) - 15g coconut oil, 3g beef tallow and 1g flaxseed oil
2. 70% diet-low unsaturated fatty acid content (80% SFA) - 21.5g coconut oil, 4.5g beef tallow and 1.3g flaxseed oil.
3. 100% diet-high unsaturated fatty acid content (20% SFA) - 7g soybean oil, 3g beef tallow, 8g olive oil and 1g flaxseed oil.
4. 70% diet-high unsaturated fatty acid content (20% SFA) - 10.5g soybean oil, 4.5g beef tallow, 11g olive oil and 1.3g flaxseed oil

[d] Energy reductions are achieve by appropriate reduction in amounts based on 100% intake group consumption.

However, recent work on the enzyme and its inhibition have demonstrated that carbohydrate induced obesity and amount of dietary oleate require increased attention. Work by Ntambi et al (*Cell Metabolism 6, 484-496, December 2007*), using triolein (synthetic triacylglycerol of only oleate fatty acids), has demonstrated that endogenously produced oleate may be the crucial to normal fatty acid and glucose metabolism. Therefore, we are amending the protocol to conform with current understanding of the SCD-1 pathway and to avoid duplicating work of other in the field. The new diets will use high carbohydrate versus high fat diets to induce obesity in the preconditioning phase and the fat component of the diet will be supplemented with either triolein or tristearin to control the fatty acid profile of the diet. The specific changes are listed in following table.

Table 2. New Experimental Diet Composition

Ingredients (based on Harlan Teklad TD.07910)	Diet fed *ad libitum* g/100g diet
Casein (vitamin free test)	19.0
DL-Methionine	0.3
Corn Starch	4.1
Sucrose	48.7
Maltodextrin	15.0
Cellulose	5.0
Corn oil[c]	0.5
Triolein or Tristearin[c]	1.5
Vitamin Mix[a]	1.0
Mineral Mix[b]	3.5
Potassium phosphate, monobasic	0.9
Calcium carbonate	0.4
Magnesium oxide	0.14
Ferric citrate	0.03
TBHQ, antioxidant	0.001
Food color	0.015
Energy[d] (kcal/100g)	360

[a] Harlan Teklad vitamin mix (CA. 40060, Harlan Teklad, WI, USA)

[b] AIN-93M mineral mix (AIN-93G-MX, TD 94049, Harlan Teklad, WI, USA)

c used to replace previous high saturated versus high polyunsaturated.

[d] Energy reductions are achieve by appropriate reduction in amounts based on 100% intake group consumption. Macronutrient breakdown is %kcal from protein, carbohydrate and fat as 19.4%, 75.6% and 5.0%, respectively. Energy restricted animals in experiment 2 will receive 70% of the total ad libitum food consumption.

Table 3. Fatty acid breakdown

Fatty Acids	**Diet with Triolein**	**Diet with Tristearin**
C6	0%	0%
C8	0%	0%
C10	0%	0%
C12	0%	0%
C14	0%	0%
C16	3%	3%
C16:1	0%	0%
C18	1%	76%
C18:1	82%	7%
C18:2	14%	14%
C18:3	0%	0%

Appendix 4: Amendment to Protocol F07-022 Approval

UNIVERSITY OF MANITOBA | OFFICE OF RESEARCH SERVICES
Office of the Vice-President (Research)

CTC Building
208 - 194 Dafoe Road
Winnipeg, MB R3T 2N2
Fax (204) 269-7173
www.umanitoba.ca/research

February 28, 2008

TO: Dr. P. Jones, Richardson Centre, 196 Innovation Drive

FROM: Dr. T. Dick, Chair, Fort Garry Campus Protocol Management and Review Committee [redacted]

RE: Amendment to Protocol F07-022

Please be advised that the amendment as documented in your application for amendment (attached), to the above noted protocol, has been approved.

TD/lh

cc: Veterinary Services
Teri Whittington, RCFFN Animal Holding Facility

Attach.

Appendix 5a: VLF-M (Triolein) Experimental Diet Information Sheet

Harlan Teklad — Custom Research Diets

TD.08241 1.5% Triolein Diet

Formula	g/Kg
Casein, "Vitamin-Free" Test	180.0
DL-Methionine	3.0
Sucrose	600.0
Corn Starch	187.84
Triolein, customer supplied	16.0
Safflower Oil, linoleic	4.0
Flaxseed Oil	1.0
Cellulose	60.0
Mineral Mix, AIN-93G-MX (94046)	36.0
Calcium Phosphate, dibasic	1.6
Vitamin Mix, AIN-93-VX (94047)	10.0
Choline Bitartrate	2.6
TBHQ, antioxidant	0.01
Blue Food Color	0.16

Footnote

A high sucrose diet with 1.5% triolein. Safflower oil and flaxseed oil are added to supply essential fatty acids (linoleic & linolenic). Formula has similarities to TD.07910.

Selected Nutrient Information [1]

	% by weight	% kcal from
Protein	17.4	19.2
Carbohydrate	68.7	75.8
Fat	2.0	5.0
Kcal/g	3.6	

[1] Values are calculated from ingredient analysis or manufacturer data

Speak With A Nutritionist

- 800-483-5523
- askanutritionist@teklad.com

Key Features

- Purified Diet
- Triolein
- Customer Supplied Ingredient
- Monounsaturated Fat Source

Key Planning Information

- *Products are made fresh to order*
- Store product at 4°C or lower
- Recommended use within 6 months
- Box labeled with product name, manufacturing date, and lot number
- Lead time:
  - 2 weeks non-irradiated
  - 4 weeks irradiated

Product Specific Information

- 1/2" Pellet or Powder (free flowing)
- Minimum order 3 Kg
- Irradiation not advised
  - Contact a nutritionist for recommendations

Options (Fees Will Apply)

- Rush order (pending availability)
- Irradiation (see Product Specific Information)
- Vacuum packaging (0.5, 1, 2, 2.5 Kg)

International Inquiry

·Outside U.S.A. or Canada·

- askanutritionist@teklad.com

Place Your Order (U.S.A. & Canada)

· Place Order · Obtain Pricing ·
· Check Order Status ·

- 800-483-5523
- 608-277-2066 facsimile
- customerservice@teklad.com

Harlan TEKLAD — P.O. Box 44220 · Madison, WI 53744-4220 · 800·483·5523 — www.tekladcustomdiets.com — Access to excellence

Appendix 5b: VLF-S (Tristearin) Experimental Diet Information Sheet

Harlan Teklad — *Custom Research Diets*

TD.08242 1.5% Tristearin Diet

Formula	g/Kg
Casein, "Vitamin-Free" Test	180.0
DL-Methionine	3.0
Sucrose	600.0
Corn Starch	187.84
Tristearin, customer supplied	16.0
Safflower Oil, linoleic	4.0
Flaxseed Oil	1.0
Cellulose	60.0
Mineral Mix, AIN-93G-MX (94046)	36.0
Calcium Phosphate, dibasic	1.6
Vitamin Mix, AIN-93-VX (94047)	10.0
Choline Bitartrate	2.6
TBHQ, antioxidant	0.01
Pink Food Color	0.16

Key Features

- Purified Diet
- Tristearin
- Customer Supplied Ingredient
- Saturated Fat Source

Key Planning Information

- *Products are made fresh to order*
- Store product at 4°C or lower
- Recommended use within 6 months
- Box labeled with product name, manufacturing date, and lot number
- Lead time:
  - 2 weeks non-irradiated
  - 4 weeks irradiated

Product Specific Information

- 1/2" Pellet or Powder (free flowing)
- Minimum order 3 Kg
- Irradiation not advised
  - Contact a nutritionist for recommendations

Footnote

A high sucrose diet with 1.5% tristearin. Safflower oil and flaxseed oil are added to supply essential fatty acids (linoleic & linolenic). Formula has similarities to TD.07910.

Options (Fees Will Apply)

- Rush order (pending availability)
- Irradiation (see Product Specific Information)
- Vacuum packaging (0.5, 1, 2, 2.5 Kg)

Selected Nutrient Information [1]

	% by weight	% kcal from
Protein	17.4	19.2
Carbohydrate	68.7	75.8
Fat	2.0	5.0
Kcal/g	3.6	

[1] Values are calculated from ingredient analysis or manufacturer data

International Inquiry

·Outside U.S.A. or Canada·

- ackanutritionist@teklad.com

Speak With A Nutritionist

- 800-483-5523
- ackanutritionist@teklad.com

Place Your Order (U.S.A. & Canada)

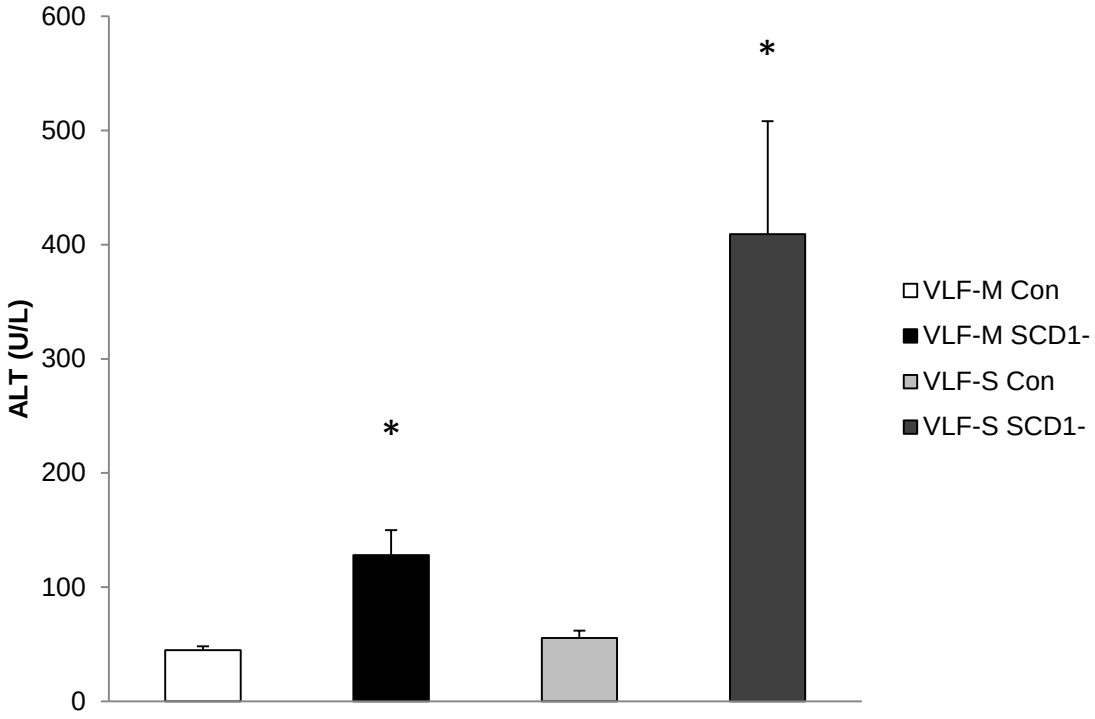
· Place Order · Obtain Pricing ·
· Check Order Status ·

- 800-483-5523
- 608-277-2066 (facsimile)
- customerservice@teklad.com

Harlan TEKLAD — P.O. Box 44220 · Madison, WI 53744-4220 · 800 · 483 · 5523
www.tekladcustomdiets.com

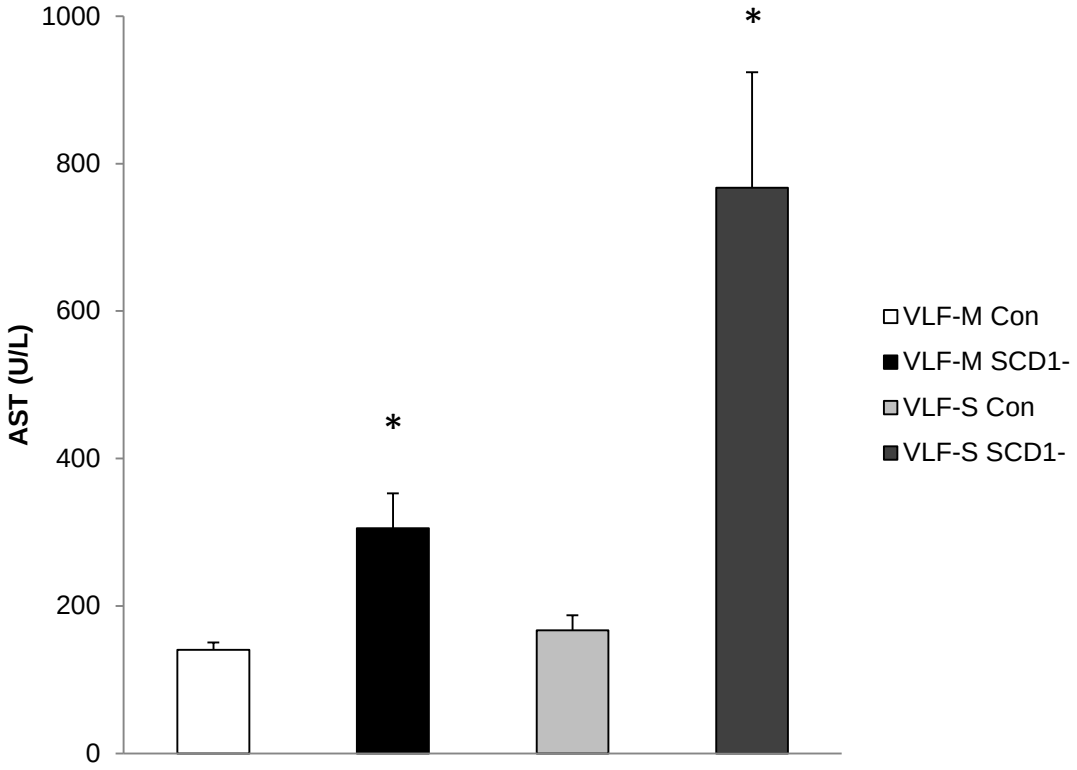
Access to excellence

Appendix 6: Alanine aminotransferase in non-fasted VLF-M and VLF-S mice



Data are presented as means ± SE (n=8/group). Statistical differences are indicated as a *, assessed at $p<0.05$.

Appendix 7: Aspartate aminotransferase levels in non-fasted VLF-M and VLF-S mice



Data are presented as means ± SE (n=8/group). Statistical differences are indicated as a *, assessed at $p<0.05$.