

**CONTRIBUTION OF DE NOVO LIPOGENESIS TO
THE PROGRESSION OF NONALCOHOLIC FATTY LIVER DISEASE**

A Dissertation

Presented to the Faculty of the Graduate School
University of Missouri, Columbia

In Partial Fulfillment
of the Requirements for the Degree:
Doctor of Philosophy

By

SYED ABDUL, MAJID MUFAQAM

Doctoral Candidate, Nutritional Sciences

University of Missouri, Columbia

Advisor: Elizabeth J. Parks, PhD

December 2020

This page is left intentionally blank

The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

CONTRIBUTION OF DE NOVO LIPOGENESIS TO
THE PROGRESSION OF NONALCOHOLIC FATTY LIVER DISEASE

presented by Syed Abdul, Majid Mufaqam,
a candidate for the degree of Doctor of Philosophy,
and hereby certify that, in their opinion, it is worthy of acceptance.

Professor Elizabeth Parks

Professor Pamela Bruzina

Professor Jill Kanaley

Professor Stephen Ball

Professor Jamal Ibdah

Assistant Professor Brian Bostick

Dedicated to my lovely wife Azra Fatima Syed

ACKNOWLEDGEMENTS

First of all, I would like to thank Almighty Allah, the God, and my parents for being ever so kind and courteous to me, and my wife for her support and patience during my doctoral training.

I express my deep sense of gratitude and sincere thanks to Dr. Elizabeth Parks for accepting me as her student during my doctoral training at the University of Missouri. I am thankful to her for her mentorship, support, and training for these five years. Dr. Parks has been instrumental in many ways during my training, including improving my writing and communication skills. My day-to-day discussions with Dr. Parks have fostered my critical thinking abilities which cannot be learned through formal training. It would not have been possible for me to accomplish these academic goals without her help at every point during my training.

I would like to thank my dissertation committee members, Dr. Jamal Ibdah, Dr. Pamela Bruzina, Dr. Jill Kanaley, Dr. Stephen Ball, and Dr. Brian Bostick for their critical input during my doctoral training and for being patient during committee meetings.

Importantly, I would also like to thank Dr. Scott Rector, his lab manager, Grace Meers, and his doctoral student, Mary Moore, for training and helping me with all the molecular work.

I would also like to extend my thanks to the Department of Nutrition and Exercise Physiology, Clinical Research Center, Division of Gastroenterology and Hepatology, the Department of Surgery, and the Department of Radiology. All the physicians and surgeons especially, Dr. Ayman Gaballah, Dr. Andrew

Wheeler, Dr. Rama Rao Ganga, Dr. Camila Manrique, and Dr. Ghassan Hammoud for their direct or indirect support during my training. Our former lab manager, Nhan Le for training me in lab procedures during my early doctoral years. My dissertation project would not have been made possible without the help of Jennifer Anderson who helped me with patient recruitment.

Special thanks to Dr. Christopher Hardin, the Chair of NEP for his exceptional efforts in advocating and supporting graduate students and making our life easier during these years of training.

This dissertation wouldn't have been made possible without financial support from the American Society for Nutrition, MU Dissertation Year Fellowship, Dr. Parks' lab resources, Dr. Rector resources at the VA, and the NEP resources.

I would also like to thank United States Customs and Immigration Services (USCIS) for granting me a student visa and allowing me to enter the United States to accomplish my academic goals.

Last but not the least, I would also like to thank my former lab mates, Miriam Jacome-Sosa, Qiong Hu, Kimberlee Bingham, and Kathy Smith, and my current lab mates, Justine Mucinski, Megan Searles, Talyia Fordham, and all the undergraduates who have helped in many ways to make me academically successful.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	ii
ABBREVIATIONS.....	v
LIST OF TABLES.....	viii
LIST OF FIGURES.....	ix
ABSTRACT.....	xi
Chapter I - Introduction and literature review.....	1
Background.....	2
Specific aims.....	24
References.....	34
Table 1.1 references.....	55
Chapter II - Contribution of de novo lipogenesis during stages of nonalcoholic fatty liver disease.....	57
Introduction.....	60
Methods.....	62
Results.....	69
Discussion.....	83
Summary.....	99
Limitations and future recommendations.....	100
Significance of the study.....	101
References.....	151
Extended methodology.....	163
Extended results.....	179
Extended references.....	185
Chapter III - First-in-class fatty acid synthase inhibitor TVB-2640 reduces hepatic de novo lipogenesis in males with metabolic abnormalities.....	189
Introduction.....	192
Methods.....	195
Results.....	204
Discussion.....	208
References.....	225
Supporting information.....	230
Chapter IV - The Tailgate Study: Differing metabolic effects of a bout of excessive eating and drinking.....	243
Introduction.....	246
Materials and methods.....	248
Results.....	256
Discussion.....	259
References.....	274
APPENDIX A - NAFLD activity score system.....	278
NAS grading sheet.....	278
APPENDIX B - Informed consent forms.....	279
Chapter II - Consent form.....	279
Chapter III - Consent form.....	286
Chapter IV - Consent form.....	296
VITA.....	305

ABBREVIATIONS

AASLD - American association for the study of liver disease
ABCG5/8 - ATP-binding cassette sub-family G member 5/8
ACAT - acetyl-CoA acetyltransferases
ACC - acetyl-CoA carboxylase
A-CoA - acetyl coenzyme A
ACS - acetyl coenzyme-A synthase
AGPAT - 1-acylglycerol-3-phosphate-O-acyltransferase
ALP - alkaline phosphatase
ALT - alanine aminotransferase
AMPK - activated protein kinase
APOB100 - apolipoprotein B100
APRI - aspartate aminotransferase to platelet ratio index
AST - aspartate aminotransferase
ATGL - Adipose triglyceride lipase
ATP - adenosine triphosphate
AUC - area under the curve
BAL - blood alcohol level
BARD - BMI, AST/ALT ratio, diabetes
BCA - BSA concentration assay
BMI - body mass index
BP - blood pressure
BrAC - breath alcohol concentrations
BSA - bovine serum albumin
BW - body weight
CAP - controlled attenuation parameter
CD36 - cluster of differentiation 36
CE - cholesterol esters
CHO - carbohydrates
CPT1 - carnitine palmitoyltransferase 1
CYP 7A/27A - cholesterol 7/27 alpha-hydroxylase
D2O - deuterated water
DAG - diacylglycerols
DDP - duration of the drinking period
DGAT - diacylglycerol acyltransferase enzyme
DHA - docosahexaenoic acid
DNL - de novo lipogenesis
DSI - Dallas steatosis index
EI - elongation index
ELOV - elongases
EPA - eicosapentaenoic acid
F/GTT - oral fructose/glucose tolerance test
FA - fatty acid
FABP - fatty acid binding protein
FAs - fatty acids

FAS/FASN - fatty acid synthase
FAST - FibroScan-AST
FATP - fatty acid transport protein
FC - free cholesterol
FFA - free fatty acids
FIB-4 - fibrosis index based on the 4-factors
FS - NAFLD fibrosis score
FSI - Framingham steatosis index
GAPDH - glyceraldehyde 3-phosphate dehydrogenase
GC - gas chromatography
GC/MS - gas chromatography/mass spectrometry
GPAT - glycerol-3-phosphate
HbA1c - hemoglobin A1C
HCC - hepatocellular carcinoma
HDL - high-density lipoprotein
HDLc - high-density lipoprotein cholesterol
HELLP - hemolysis, elevated liver enzymes, low platelet count
HSL - hormone-sensitive lipase
HMG-CoA - β -Hydroxy β -methylglutaryl-CoA
HMGCR - β -Hydroxy β -methylglutaryl-CoA reductase
HMGCS - β -Hydroxy β -methylglutaryl-CoA synthase
HOMA-IR - homeostatic model assessment of insulin resistance
HPLC/MS/MS - high-pressure liquid chromatography and mass spectrometry
HSI - hepatic steatosis index
IHTG - intrahepatic triglycerides
IL6 - interleukin 6
JNK - c-Jun N-terminal kinase
LD - linoleate dilution
LDL- low-density lipoprotein
LDLc - low-density lipoprotein cholesterol
LDL-R - low-density lipoprotein receptor
LFS - NAFLD liver fat score
MAG - monoacylglycerol
MGAT - monoacylglycerol acyltransferase enzyme
MIDA - mass isotopomer distribution analysis
MR - alcohol metabolism rate
MRE - magnetic resonance elastography
mRNA - messenger ribonucleic acid
MRS - magnetic resonance spectroscopy
MTP - mitochondrial trifunctional protein
MTTP - microsomal triglyceride transfer protein
MUFA - monounsaturated fatty acids
NADPH - nicotinamide adenine dinucleotide phosphate.
NAFLD - nonalcoholic fatty liver disease
NAS - NAFLD activity score
NASH - nonalcoholic steatohepatitis

NCEH1 - neutral cholesterol ester hydrolase 1
NDSR - nutrition data systems for research
NEFA - nonesterified fatty acids
NPC1L1 - Niemann Pick C1-like 1
NRF2 - nuclear factor erythroid 2-related factor 2
PCR - polymerase chain reaction
PDAT - Phospholipid:diacylglycerol acyltransferase
PGC-1 α - PPAR- γ coactivator 1-alpha
Pi - phosphate
PL - phospholipids
PPAR- α - peroxisome proliferator-activated receptors α
PPAR- γ - peroxisome proliferator-activated receptors γ
PPH-1 - phosphohydrolase-1
PRO - proteins
PUFA - polyunsaturated fatty acids
qRT-PCR - quantitative real-time polymerase chain reaction
QUICKI - quantitative insulin-sensitivity check index
RNA - ribonucleic acid
ROS - reactive oxygen species
SCD index - desaturation index
SCD-1 - stearyl-CoA desaturase-1
SFA - saturated fatty acid
SOAT2 - sterol O-acyltransferase 2
SPSS - statistical package for the social sciences
SREBP1c - sterol regulatory element binding protein 1c
SREBP2 - sterol regulatory element binding protein 2
TAG - triacylglycerols
TBW - total body water
TLC - thin layer chromatography
TNF- α - tumor necrosis factor α
TPB - time to peak BAL
TRL - triglyceride-rich lipoprotein
VCTE - vibration controlled transient elastography (stiffness)
VLDL - very low-density lipoprotein

LIST OF TABLES

Chapter 1

Table 1.1: Summary of gene expression in NAFLD and NASH.....	25
--	----

Chapter 2

Table 2.1: Primer sequences for SYBR Green quantitative real-time PCR	103
Table 2.2: Subject characteristics.....	105
Table 2.3: Liver-related measurements between the groups.....	107
Table 2.4: Analytical and biochemical measurements between the groups	108
Table 2.5: Lipid subclass analysis in the liver and the TRL particle across the disease severity.....	110
Table 2.6: Calculated DNL indices across disease severity	113
Table 2.7: Correlation analysis of DNL indices with isotopic measurements of DNL	115

Chapter 3

Table 3.1: Subject characteristics at screening	213
Table 3.2: Biochemical measurements and substrate oxidation before and after 10 days TVB-2640 treatment.....	214

Chapter 4

Table 4.1: Dietary intake in a subset of subjects' ad libitum days for comparison to the study day	265
Table 4.2: Baseline subjects characteristics.....	266

LIST OF FIGURES

Chapter 1

Figure 1.1: Fatty acid synthesis	26
Figure 1.2: Triglycerides synthesis	28
Figure 1.3: Cholesterol synthesis	29
Figure 1.4: Lipid secretion and storage	30
Figure 1.5: Fatty acid oxidation.....	31
Figure 1.6: Measured and predicted DNL.....	32
Figure 1.7: Labeling of the DNL pathway	33

Chapter 2

Figure 2.1: Consort flow	118
Figure 2.2: Study design.....	119
Figure 2.3: NAFLD activity score system and histology.....	120
Figure 2.4: NAFLD activity score and fibrosis score in each group	121
Figure 2.5: NAFLD calculators and histological scoring	122
Figure 2.6: Lipid content of the liver and the TRL particle	123
Figure 2.7: Liver lipids concentrations across NAFLD stages	124
Figure 2.8: De novo lipogenesis in liver-TAG	125
Figure 2.9: De novo lipogenesis in the other liver lipids.....	127
Figure 2.10: DNL in NAFLD stages	128
Figure 2.11: DNL in the TRL-TAG	129
Figure 2.12: DNL in the TRL-CE.....	130
Figure 2.13: Relationships between liver and TRL percent DNL	131
Figure 2.14: mRNA expression of key genes involved in the DNL pathway	132
Figure 2.15: Expression of key proteins involved in the DNL pathway	133
Figure 2.16: Correlation analysis of DNL and FASN	134
Figure 2.17: Correlation analysis of labeled DNL vs DNL gene expression	135
Figure 2.18: Correlation analysis of DNL and FASN	136
Figure 2.19: mRNA and protein expression of key enzymes involved in TAG metabolism	137
Figure 2.20: mRNA and protein expression of key enzymes involved in FA transport and oxidation	138
Figure 2.21: mRNA expression of key genes involved in cholesterol metabolism	139
Figure 2.22: DNL FAs contribute more toward storage	140
Figure 2.23: Percent DNL in other FA species	141
Figure 2.24: Absolute DNL in other FA species.....	142
Figure 2.25: Total absolute DNL FAs in the liver	143
Figure 2.26: Absolute DNL contribution in each FA.....	144
Figure 2.27: Insulin resistance in the severity of the disease	145
Figure 2.28: Insulin resistance, DNL, and DNL enzymes	146
Figure 2.29: FA Composition in the liver and TRL lipids.....	147
Figure 2.30: FA subclass, DNL enzymes, and NAFLD progression	148

Figure 2.31: DNL vs NEFA associations with metabolic characteristics and cholesterol pathway in NAFLD	149
Figure 2.32: DNL contribution to NAFLD progression	150

Chapter 3

Figure 3.1: Consort flow	215
Figure 3.2: Overall study design and in-patient protocol.....	216
Figure 3.3: Pharmacokinetics and plasma concentration AUC after 10d of dosing	218
Figure 3.4: Changes in liver enzymes, plasma lipids, and liver fat	219
Figure 3.5: Changes in DNL and correlation analysis between TVB-2640 AUC, DNL, and liver fat.....	221
Figure 3.6: Changes in liver enzymes, plasma lipids, and liver fat	223

Chapter 4

Figure 4.1: Study consort flow diagram and protocol timeline	268
Figure 4.2: Breath alcohol concentrations, subjective intoxication, plasma insulin and metabolites	269
Figure 4.3: Changes in DNL and liver fat.....	270
Figure 4.4: Group differences in DNL, TRL-TG, food intake, and breath alcohol concentrations	272
Figure 4.5: Plasma insulin and blood metabolites, and fasting whole-body, fat oxidation	273

ABSTRACT

This dissertation is focused on understanding the biochemical pathway of de novo lipogenesis (DNL) in humans and how changes in DNL can alter disease states, particularly nonalcoholic fatty liver disease (NAFLD). This document's first chapter presents a review of the literature, while the second chapter focuses on investigating the contribution of DNL to the progression of NAFLD. The main outcome of this study was that as disease severity progressed, hepatic DNL increased in a stepwise fashion until fibrosis was significant, at which time DNL was found to be reduced. Conclusions from isotopic labeling of liver were mirrored by data from protein and gene expression studies which pointed toward mechanisms of promoting both fat storage and decreased fatty acid oxidation. In the third chapter, data are presented on the effects of pharmacological inhibition of DNL, which lowered both liver fat (from 11.8% to 10.3%) and liver enzymes (from 29 to 22 U/L). In this study, different subjects received different doses (50, 100, and 15 mg/d) and within the high dose group, DNL was reduced maximally by 75% which resulted in a 5% reduction in liver fat and a 36% reduction in liver enzymes. Lastly, this dissertation's fourth chapter presents data from an investigation in which acute overconsumption of food and alcohol increased liver fat only in individuals whose DNL was stimulated by this treatment but not in individuals with unchanged DNL. The variability in response between subjects was surprising and suggested that for some people, overconsumption of carbohydrates may have greater lipogenic effects than excess alcohol. Future studies should identify the factors that govern this response.

In summary, the combined data from these studies highlight the significance of the DNL pathway in promoting increased fat storage in the liver. This conclusion is supported by independent observations of both the negative effects of increased DNL on the liver and the benefits of reducing flux through this pathway to improve liver health. Both dietary and pharmacologic approaches to reduce DNL should be the focus of future treatment of NAFLD.

Chapter I - Introduction and literature review

BACKGROUND

Nonalcoholic fatty liver disease

Nonalcoholic fatty liver disease (NAFLD) is a condition with a spectrum of diseases ranging from increased liver fat ($\geq 5.5\%$ of liver volume via imaging or histology), inflammation, and cell destruction in its more advanced forms (1-4). Factors that would differentiate NAFLD from other types of liver diseases include fatty liver, the absence of excessive alcohol intake defined as 14 drinks per week for women or 21 drinks for men (5, 6), no use of steatogenic medications (e.g., valproate, anti-retroviral medicines, amiodarone, methotrexate, tamoxifen, corticosteroids), absence of hereditary disorders (Wilson's disease, lipodystrophy, abetalipoproteinemia, Reye's syndrome, HELLP syndrome, and inborn errors of metabolism like LCAT deficiency, cholesterol ester storage disease, and Wolman disease), and other conditions like acute fatty liver of pregnancy, hepatitis C, starvation, parenteral nutrition (5, 7).

Stages of NAFLD

The general development of NAFLD occurs in four stages (1-4, 8, 9). The first stage, called hepatic steatosis, begins with increased accumulation of intrahepatic triacylglycerols (IHTG) (4, 6, 10-14), primarily observed due to abnormal hepatic lipid metabolism in the presence or absence of obesity (15-24). This initial stage of NAFLD, when left untreated, can progress in approximately 20% of the cases to a more advanced stage called nonalcoholic steatohepatitis (NASH) characterized by increased inflammation, cellular stress, and apoptosis

(6, 10, 12, 25-30). Recently, NASH has been reported as a rapidly growing cause of hepatocellular carcinoma (HCC) in patients awaiting liver transplantation (18). Of these, roughly 13-25% of the patients with NASH can advance to the third stage of NAFLD, termed fibrosis, involves scarring of the liver tissue (28) which can result in permanent damage subsequently leading to cirrhosis of the liver in 2-12% of the patients (stage 4), and eventually to HCC (1, 4, 6, 8, 14, 24, 28, 31). Clinically, detection of NAFLD during the early stages remain a crucial treatment goal (7). Once the disease reaches stage 4 - cirrhosis, the primary treatment option available is liver transplantation, even though other therapies like antibiotics, antivirals, beta-blockers, and angiotensin-converting enzyme inhibitors are currently being utilized to alleviate cirrhosis-associated complications (32). Therefore, there is a serious need to understand the mechanism of NAFLD's progression to its more advanced stages i.e. NASH, fibrosis, and eventually to irreversible conditions such as cirrhosis and HCC (33, 34).

Prevalence

Currently, almost a quarter of the population in the United States and around the world have characteristics of NAFLD (19, 35) and these rates are increasing (36). Paralleling increases in obesity, increases in NAFLD are significantly contributing to the growing burden of chronic liver disease in the United States and worldwide (16, 19, 35-40). As expected, given the increased prevalence of NAFLD, NASH has increased 2.0-2.5 fold over the past few years (19, 41) and is strongly

associated with liver-related morbidity (14, 42). Moreover, NASH was projected to become the second most common cause of liver transplantation (33).

Pathogenesis

As stated above, the early pathogenesis of NAFLD and NASH essentially includes the accumulation of fat in the liver (4, 6, 10-14, 43). This key feature of NAFLD has been utilized, in several human studies, as an indicator of the presence of NAFLD (44-54). The characterization of patients as having NAFLD by using only elevated liver enzymes for diagnosis is somewhat imprecise since the AASLD advocates a diagnosis through imaging or histological evaluation of liver biopsy (5). The understanding of the development of NAFLD is based on data from several rodents and human studies (1, 4, 8, 10-12, 55-57). With excess energy intake (positive energy balance), nutrient flux overloads the liver metabolic pathway (58). Regardless of the source of energy (carbohydrate, fat, or protein), excess carbons are stored in the liver in the form of fats either directly (dietary fats) or through de novo lipogenesis (DNL) which is defined as the synthesis of fatty acids (FAs) from carbohydrate (59-62). Only one study to date has shown evidence of DNL made from amino acids (63). While the presence of dietary FAs has been recognized as promoting NAFLD (64-67), dietary carbohydrates have been shown to play a major role in the development of fatty liver (68, 69). Additionally, studies have investigated the role of cholesterol metabolism in NAFLD and reported increased free cholesterol (FC) in NAFLD/NASH patients (70, 71).

Lipid synthesis and oxidation in NAFLD

De novo lipogenesis - As shown in **figure 1.1a**, DNL is the process of liver synthesis of FAs from carbohydrates (61, 62). Sterol regulatory element binding protein (SREBP)-1c, a master regulator of the DNL pathway, is activated in the presence of insulin (72). Four key enzymes are involved in the synthesis of FAs are acetyl coenzyme-A carboxylase (ACC), fatty acid synthase (FASN), stearoyl-CoA desaturase-1 (SCD-1), and a set of enzymes called elongases (ELOV) (73). As shown in **figure 1.1b**, acetyl coenzyme A (Ac-CoA) is the starting molecule in FA synthesis. In the presence of ACC and biotin, Ac-CoA undergoes irreversible carboxylation to produce malonyl-CoA. These two molecules go through multiple cycles in the protein complex (typically eight cycles), FASN, which primarily produces a 16-carbon FA from eight Ac-CoAs. The primary product of DNL is saturated FA (**figure 1.1a**). Once the FAs are synthesized through the DNL pathway, they may be desaturated via SCD-1 and elongated via ELOV enzymes. These newly-made FAs are then used for energy (ATP synthesis), the synthesis of triacylglycerol (TAG), phospholipids (PL), ceramides, and are also esterified to cholesterol to make cholesterol esters (CE) (**figure 1.1c**) (74). The initiating events of NAFLD involve insulin resistance as a result of excess nutrient intake and positive energy balance. Due to hyperinsulinemia, SREBP1c, and the subsequent key enzymes of the DNL pathway, i.e., ACC, FASN, and SCD-1, have been reported to be increased in both animal and cell culture studies of NAFLD (49, 75-84). Another regulator of hepatic DNL is carbohydrate response element binding protein (ChREBP), primarily activated in the fed state or during

hyperglycemia (85-88), and has been shown to stimulate the transcription of enzymes or directly increased the activity of these enzymes (86, 89, 90). Given its role in glucose and lipids homeostasis (91-94), downregulation of liver ChREBP in mice prevented steatosis from high-carbohydrate feeding but increased hepatic glucose production, and decreased FA oxidation and insulin sensitivity (95), whereas insulin sensitivity was improved in the *ob/ob* mouse model with similar treatment (86, 96). Although inhibition of ChREBP has been shown to reduce fructose-induced DNL and increased TAG content (97), complications like fructose intolerance, diarrhea, irritable bowel syndrome, and cholesterol-induced hepatotoxicity were observed (97-100). By contrast, adenoviral overexpression of ChREBP in mice increased IHTG, steatosis grade, and insulin sensitivity (90). In obese individuals, a positive relationship was observed between liver mRNA expression of ChREBP β and insulin resistance and steatosis, however, in NASH patients, a negative relationship was observed between ChREBP β and insulin resistance (86, 90, 101). Although it is unclear why these discrepancies were observed between studies, Abdul-Wahed et al suggested that these differences could be due to the influence of variations in genetic, dietary, or environmental factors (86). Overall, DNL appears to be activated by insulin-dependent and independent pathways via SREBP1c and ChREBP pathways (102) and contributes to almost 26% of the total FAs in the liver lipid pool (103). Early studies from the Parks lab have established that elevated DNL is the unique, early event distinguishing subjects with NAFLD compared to subjects who were equally-obese with low intrahepatic

triacylglycerols (IHTG) (44, 103-105). Other studies have also reported similar findings i.e., higher DNL in individuals with high liver fat compared to healthy/individuals with normal liver fat (62, 72, 106-108). Despite increases in NAFLD prevalence over the last two decades in the US and worldwide (18, 19, 35), no drug therapies are currently approved for NAFLD treatment. Given the contribution of DNL in NAFLD pathogenesis (44, 45, 89, 105, 109), scientists from the pharmaceutical industry have been investigating drugs (110-113) that inhibit the DNL pathway (46-51, 114). These pharmacological inhibitors of DNL enzymes have been shown to reduce IHTG in NAFLD patients and in individuals with the characteristics of metabolic syndrome that put them at risk for developing NAFLD (46, 47, 50, 51, 82, 114).

Triglycerides synthesis - As shown in **figure 1.2**, three sources of liver FAs, those synthesized via DNL, those released from adipose tissue (nonesterified FA, NEFA), or obtained from the diet are converted into fatty acyl-CoA. This fatty acyl-CoA is added to glycerol-3-phosphate by glycerol-3-phosphate acyltransferase (GPAT) to form lysophosphatidate. A second fatty acyl-CoA is added in the presence of 1-acylglycerol-3-phosphate-O-acyltransferase (AGPAT) to form phosphatidate. Phosphatidate is then converted into diglycerides (DAG) in the presence of phosphohydrolase-1 (PPH-1). DAGs can also be synthesized from monoglyceride (MAG) in the presence of monoacylglycerol acyltransferase (MGAT, not shown in the figure). Lastly, a fatty acyl-CoA is added to DAG in the presence of diacylglycerol acyltransferase enzyme (DGAT) to form TAG. These

synthesized TAG are then either incorporated into lipoproteins, which are secreted into the bloodstream, utilized for energy, or stored in the tissue for future utilization (115). Hypertriglyceridemia, one of the hallmark characteristics of patients with NAFLD and NASH (15, 17, 19, 20, 116), is an indicator of increased storage of TAGs in the liver (the first step in the development of NAFLD) (12, 17, 117, 118). Some pharmaceutical industries are currently working on developing and testing a pharmacological inhibitor of DGAT enzymes to decrease TAG synthesis and eventually IHTG in patients with NAFLD (Clinicaltrials.gov# NCT01064492).

Cholesterol synthesis and esterification - As shown in **figure 1.3**, during excess nutrient intake, in addition to DNL, two molecules of Ac-CoA can also be catalyzed into acetoacetyl-CoA in the presence of Ac-CoA acetyltransferases (ACAT). Acetoacetyl-CoA, with another Ac-CoA, becomes β -hydroxy β -methylglutaryl-CoA (HMG-CoA), a key molecule in the cholesterol synthesis pathway, which undergoes several steps to produce mevalonic acid in the presence of HMG-CoA reductase (HMGR), squalene, and subsequently cholesterol (119, 120). The synthesized cholesterol can be used for bile acid synthesis, exported via ATP-binding cassette sub-family G member 5/8 (ABCG5/8), or esterified to make CE via acyl-coenzyme A: cholesterol acyltransferase 2 (ACAT2 or SOAT2) enzyme and either exported via very low-density lipoprotein (VLDL) particle or stored in the liver for later utilization (120). Compared to healthy individuals, NAFLD patients exhibit elevated plasma total

cholesterol and low-density lipoprotein (LDL) cholesterol, and lower high-density lipoprotein (HDL) cholesterol concentrations (15, 17, 19, 20, 22, 116). The master regulator of the cholesterol synthesis pathway is SREBP-2 (120) which undergoes several molecular steps to activate HMGCR, a key step in the cholesterol synthesis pathway (120). The expression of SREBP-2 activity has been reported to be higher in NASH patients compared to steatotic patients (121). A study conducted by van Rooyen et al in obese diabetic mice reported that the increased expression of SREBP-2 with a high-fat diet resulted in increased accumulation of free cholesterol (FC) in *flox/flox* mice compared to wild-type (122), and this increased FC resulted in the development of NASH and fibrosis (122). These findings were primarily attributed to increased uptake of cholesterol through the LDL-receptor (LDL-R), decreased utilization for bile synthesis, and liver reduced export (122, 123). Additionally, these mice exhibited higher esterification of cholesterol which was also supported by increased SOAT2 activity (reported as ACAT2 in the publication). Further, Min et al examined human liver samples and reported higher concentrations of FC in NAFLD and NASH patients compared to healthy individuals (70). Min et al reported that the higher FC concentrations observed in NAFLD and NASH patients were due to increased synthesis (i.e., HMGCR activity) and not through increased LDL-R uptake, or decreased utilization for bile synthesis and export (70). Given that the past data support the role of cholesterol in the development of NAFLD (124, 125), and the more severe form of NASH, by promoting inflammation and fibrosis in the liver cells (126), reducing cholesterol has been

recommended as a strategy to treat NAFLD (127). In the study conducted by Min et al (70), the mRNA expressions of SREBP2 and HMGCR were significantly lower in the patients' liver samples who were using statins, compared to patients who were not using statins. Further, in obese diabetic mice, pharmacological inhibition of cholesterol synthesis and absorption by inhibiting HMGCR and Niemann Pick C1-like 1 (NPC1L1) proteins (using atorvastatin and ezetimibe,) reversed fibrotic NASH (128).

Lipid secretion and storage - As shown in **figure 1.4**, FAs synthesized via DNL, those originating from adipose tissue (i.e., NEFA), and coming from dietary fats, are either oxidized for energy (discussed later) or utilized for the synthesis of TAG and PL using glycerol 3-phosphate as the backbone, and esterified to cholesterol coming from the dietary and endogenous sources (74). These lipids are then stored in the liver in the form of lipid droplets or exported out via the VLDL secretion into the circulation (118, 120, 125). Lipids are incorporated into a droplet via DGAT2 and are later utilized via lipolysis, whereas, DGAT1 promotes the incorporation of lipids into the VLDL particle, in the presence of apolipoprotein-B100 (apoB100) (118, 129, 130). An in vivo study conducted in healthy males demonstrated that insulin infusion, which led to decreased NEFA reduced VLDL synthesis and secretion (131). However, these suppressive effects of insulin appeared to be reduced in subjects with insulin resistance eventually leading to higher VLDL synthesis and secretion (132). A study conducted in hamsters suggested that in insulin resistance, overproduction of

apoB100 observed was likely due to increased expression of microsomal triacylglycerol transfer protein (MTTP), a greater supply of hepatic neutral lipids, and reduced apoB100 degradation - leading to increased rates of VLDL assembly and secretion (133, 134). Courtesy exists as to whether the fatty liver is due to a suppression of VLDL-TAG secretion or, or due to increased VLDL-TAG secretion, that is still insufficient to elevate liver lipid synthesis. In a study conducted by Donnelly et al, FAs were isotopically labeled before a medically indicated liver biopsy to identify the sources of the liver and VLDL TAG. the percentage of VLDL-TAG accounted for was shown to be associated with the percentage of IHTG accounted for in NAFLD patients (103). In other words after five days of infusing and feeding FA isotopes, these subjects whose liver became most labeled where the same subjects whose VLDL became most labeled. The contributions of DNL, NEFA and dietary FAs were similar between liver and VLDL-TAG. Additionally, insulin-mediated suppression of VLDL kinetics was impaired in NAFLD patients, resulting in higher concentrations of VLDL particles and apoB100 in the patients with high IHTG compared to individuals with low IHTG (44, 135). Moreover, NAFLD patients with high IHTG also failed to suppress VLDL and apoB100 release during a euglycemic hyperinsulinemic clamp suggesting hepatic insulin resistance (135). These responses have been reported as early pathophysiological manifestations in NAFLD patients, primarily due to increased TAG synthesis from non-systemic FAs (i.e. DNL) (12, 44). However, recently, a study conducted by Lytle et al in morbidly obese patients undergoing bariatric surgery measured VLDL kinetics in low IHTG and high IHTG

groups, and reported that the increased IHTG was likely due to a decreased VLDL secretion rather than increased liver uptake of plasma NEFA (136). However, the VLDL secretion rates observed by Lytle et al were still higher than those observed previously in class I obese individuals (137). Further, results from Lytle et al were supported by a study conducted by Fujita et al who reported lower VLDL synthesis and secretion rates in NASH patients compared to NAFLD patients (138). Fujita et al also reported that the lower VLDL secretion in NASH patients was due to inefficient VLDL synthesis and reduced mRNA expression of both apoB100 and MTP (138) - a protein involved in the incorporation of TAG into VLDL particle (139). Charlton et al quantitated apoB100 synthesis rates and reported lower rates in NASH patients compared to lean and obese individuals suggesting a role of apoB100 synthesis in the development of NASH (140). By contrast, in a non-NAFLD study, Smith et al found greater VLDL secretion in obese men (141). Combined, these data suggest that VLDL synthesis and secretion may play an important role in the development of NAFLD and NASH but that, VLDL secretion observed in NAFLD/NASH may be insufficient to keep up given the higher TAG synthesis rates in these patients.

FA oxidation - As shown in **figure 1.5**, in addition to FAs from DNL, diet, and NEFA, FAs are also derived from the catabolism of TAG and hydrolysis of CE, and can undergo oxidation by the cell when energy is needed (142). Carnitine palmitoyltransferase 1 (CPT1) is a key outer-membrane mitochondrial enzyme that is involved in the transfer of FAs to facilitate their oxidation via β -oxidation

pathway (143-146). Once FAs enter mitochondria, they undergo four steps of β -oxidation (oxidation, hydration, oxidation, and thiolysis) to reduce two-carbons from a FA (147, 148). The last three steps take place on a protein complex called mitochondrial trifunctional protein (MTP) (149-151). Typically, for palmitate (16 carbon FA), the FA oxidation step undergoes eight cycles for complete oxidation of palmitate and produces eight molecules of Ac-CoA. The role of FA oxidation in the development of NAFLD has been discussed earlier in the literature and conflicting results were reported (13, 17, 152-162). However, the majority of these studies measured β -hydroxybutyrate, as an indicator of β -oxidation (17, 152-154, 157, 161). In one study, Kotronen et al reported no differences in β -hydroxybutyrate between NAFLD patients and healthy subjects (161). By contrast, many other studies reported a reduction in FA oxidation in NAFLD as measured by the enzymatic activity of CPT1 and PPAR- α , plasma β -hydroxybutyrate, ^{13}C -NMR, or breath $^{13}\text{CO}_2$ (13, 156-160, 163). A study conducted by Croci et al (157) reported decreased β -oxidation, as indicated by lower plasma β -hydroxybutyrate concentrations, in NAFLD patients compared to healthy controls. Further, a study conducted by Fletcher et al also reported reduced ketone synthesis in NAFLD, however, when they calculated FA oxidation by ^{13}C -NMR (β -oxidation), no differences were observed between NAFLD patients and healthy controls (158) suggesting ketone synthesis may not be a good indicator of FA oxidation. Recently, a study conducted by Naguib et al measured FA oxidation using a breath measurement and reported reduced dietary FA oxidation in NAFLD patients compared to healthy individuals (163).

Fletcher et al suggested that peripheral NEFA are not the sole factor responsible for promoting lipid catabolism in NAFLD patients given β -oxidation was not different despite reduced NEFA (158). While FA oxidation in NAFLD was shown to be regulated by the MTP protein (164), in the majority of the studies, decreased FA oxidation was attributed to an increased DNL (165-167). This is because an intermediate molecule, malonyl-CoA, synthesized in the DNL pathway inhibits CPT1 activity (165-167) which prevents the entrance of FAs for their oxidation in the mitochondria including those derived from the plasma NEFA pool or the diet (10, 13, 157) (**figure 1.5**).

Molecular changes in NAFLD

As shown in **table 1.1**, Several gene expression studies conducted on human hepatocytes obtained from NAFLD patients or bariatric surgery patients have shown increased gene expression of key enzymes involved in the DNL pathway (168-173). Among these, ACC isoforms were increased at least 8-fold in patients diagnosed with NAFLD, compared to patients with healthy livers (168, 169, 171). Similarly, the expression of hepatic FASN was significantly increased by at least one to five-fold in patients diagnosed with NAFLD compared with control subjects (168-170, 172). Expression of the genes for stearoyl-CoA desaturase, SCD-1, was also increased by 9-fold in NAFLD patients (168). SREBP1c, a master regulator and a key transcription factor involved in the activation of the DNL pathway (72, 119, 174), primarily activated by insulin, was also significantly increased in patients with NAFLD compared to non-NAFLD controls (72, 119,

169, 172, 175). Besides DNL enzymes, NAFLD patients also exhibited increased expression of genes responsible for fatty acid uptake (fatty acid transport protein (FATP)/fatty acid binding protein (FABP) (168, 169, 173, 176, 177) and CD36 (168, 173, 177), TAG synthesis [DGAT2 (171) and MTTP (168, 169, 178)], and VLDL kinetics (apoB100) (168, 169, 178). The FA oxidation genes, peroxisome proliferator-activated receptors (PPAR- γ) (176) and CPT1 (168, 176), were increased, and PPAR- γ coactivator 1-alpha (PGC-1 α) was decreased (179). However, results from our collaborator have found decreased FA oxidation genes in patients with NAFLD (Moore et al, unpublished dissertation work). Further, compared to NAFLD patients, NASH patients showed decreased expression of PPAR- α , apoB100, and MTTP, but no differences in SREBP1, FASN, DGAT1/2, and FAT/CD36 (138, 180, 181). Patients with cirrhosis and HCC had higher expression of CD36 and lower expression of PPAR- α (180). However, whether the changes observed in the gene expression will be in line with the results from the direct measurement of DNL (isotopically) in patients with NAFLD is unknown. Further, how the expression of these genes changes with the progression of the disease requires investigation.

Various mechanisms by which increased DNL may worsen the severity of NAFLD

Given that the NAFLD occurs in different stages ranging from steatosis to cirrhosis (1, 4, 8), to date, the contribution of DNL at each stage of NAFLD has not been measured. This is the topic of chapter II of this dissertation. The level

of DNL measured depends on the duration of labeling the pathway with isotope by IV or oral administration. In humans, Lambert et al have shown that circulating concentrations of VLDL-TAG significantly predict the magnitude of IHTG and that DNL primarily contributes to the increase in plasma VLDL-TAG (44), but whether the pathway plays a role in the disease progression is still unknown. Based on the previous literature (44, 61, 103), as shown in **figure 1.6a**, for healthy individuals, the mean value of fasting DNL has been found to be around 8% (44-46, 48, 61, 62, 104, 106, 107, 182-188). In obese individuals with characteristics of metabolic syndrome, putting them at risk for NAFLD, DNL was around 10% (51, 61, 107, 184, 188, 189), whereas, in obese hyperinsulinemic patients, DNL was ~12% (107, 184). In NAFLD patients, DNL is measured to be 27% (45, 62, 103, 188) whereas in NASH patients, DNL was 34% (44, 46, 48). The primary product of DNL is the saturated FA, palmitate, which in cell culture has been shown to significantly contribute to oxidative stress and inflammation (190), and thus one would predict that activation of this pathway increases the severity of NAFLD. Recent rodent data showed that the upregulation of the DNL through dietary supplementation of sucrose/fructose exacerbated the hepatotoxic effects of excess dietary FAs (62, 191). Furthermore, increased expression of key enzymes in the DNL pathway like SREBP1c, ACC, FASN, and SCD-1 has been observed in patients with hepatic steatosis and NASH (168-171, 178). In contrast, inhibition of the DNL through pharmacological inhibition has been shown to alleviate hepatotoxicity both in animals and humans (46, 47, 49-51, 75-82, 114).

The activation of the DNL pathway with feeding, using a high-carbohydrate diet resulted in increased insulin secretion which activates SREBP1c (83, 119, 175). Additionally, in cell culture studies, excess glucose and insulin activate ACC and FASN (192, 193) while others have shown the effect of greater stimulation of DNL with fructose (62, 182). Given the high prevalence of metabolic syndrome and insulin resistance in NAFLD patients (22), increased activity of the DNL enzymes would be expected in these individuals. Evidence supporting this concept includes the fact that hyperinsulinemia is commonly observed in patients with NAFLD (194). In patients with simple steatosis, hepatic insulin clearance is decreased resulting in a mild increase in IHTG (194). This defect in insulin clearance has been observed at the whole-body level in patients with NASH resulting in much higher levels of circulating plasma insulin concentrations leading to a greater increase in IHTG (194). However, whether insulin concentrations increase with each stage of NAFLD is unknown (**figure 1.6b**). Based on the evidence from gene expression analysis performed directly in human liver biopsies, showed significantly higher expression of SREBP1c, ACC, FASN, and SCD-1 in NAFLD patients (168-171), the DNL enzymes activity are expected to be more pronounced in severe stages of the disease. No studies have measured the expression of these enzymes at different stages of NAFLD. However, in preliminary data presented in the abstract from AASLD, no difference was observed in the fractional DNL based on fibrosis status in NAFLD patients (48) suggests that DNL may remain the same in the more severe form of the disease leading to a non-linear relationship with NAFLD activity score (**figure**

1.6c). Further, at the molecular level, increased expression of genes contributing to DNL may be significantly associated with an increased NAS score as assessed histologically.

Therefore, based on the isotopic DNL data in different conditions (obese, metabolic syndrome, and hyperinsulinemia), DNL, assessed across the spectrum of NAFLD is a big goal. These measurements should be performed in tandem with histologic scoring using the standardized NASH Clinical Research Network scoring system (195, 196).

Cholesterol in NAFLD severity - With regard to the relationship between cholesterol and NAFLD severity, hyperlipidemia is commonly observed in patients with NAFLD (15, 17, 19, 20, 116). A lipidomic analysis conducted by Puri et al compared liver tissue from patients with healthy livers, from NAFLD and NASH patients, and reported no differences in the liver-CE concentrations between these three groups. However, in a different cohort, higher FC in NASH patients was found compared to healthy individuals (71). Additionally, Puri et al reported higher circulating levels of total cholesterol (TC) concentration in both NAFLD and NASH patients compared to healthy individuals. Since no differences were observed for LDLc and HDLc between groups, the higher TC was likely to increased cholesterol in VLDL and IDL remnants (71). In a study conducted by Min et al, no differences were observed in circulating levels of TC but higher concentrations of LDLc were observed in both NAFLD and NASH patients, and lower levels of HDLc were observed in the NASH group compared

to healthy individuals (70). Moreover, Min et al performed an analysis of gene and protein expression of key enzymes involved in the cholesterol pathway and speculated that with disease severity increased synthesis (HMGCR expression increased), decreased uptake (through LDL-R), and decreased utilization for bile synthesis (CYP 7A/27A) or transport (ABCG1/8) may have increased total FC. Importantly, Min et al also reported a linear relationship between HMGCR and NAFLD activity score (NAS) score indicating cholesterol synthesis is increased with NAFLD severity. However, Min et al did not report total FC content in the liver and its association with NAS score, therefore, the true relationship between FC and disease is unknown.

The interplay between DNL and cholesterol pathway - SREBP2, a master transcriptional regulator of the cholesterol pathway, has been shown to be required for the production of an endogenous sterol ligand required for the activation of SREBP1c (174). These data and higher circulating plasma concentrations of TC and TAG in NASH patients, with increased expression of SREBP-2, suggests a link between the DNL and cholesterol synthesis pathways (121, 174). Carroll et al related acetoacetyl-CoA as a link between both pathways (26). In the DNL pathway, Ac-CoA and malonyl-CoA fuse to form acetoacetyl-CoA (represented as β -ketoacyl-ACP in **figure 1.1b**), whereas, in the cholesterol pathway, two molecules of Ac-CoA produce acetoacetyl-CoA (**figure 1.3**). Further, Carroll et al suggest that the acetoacetyl-CoA produced by the FASN enzyme in the DNL pathway can be used for cholesterol synthesis (26).

Moreover, positive relationships observed between percent DNL and circulating levels of total cholesterol and LDLc further support this relationship (189).

Surprisingly, pharmacological inhibition of the DNL enzymes reduces total cholesterol, LDLc, and HDLc both in animal models and human studies (46, 47, 49-51, 76, 78). Based on this information, both DNL and cholesterol pathways (measured isotopically, using enzymatic assays, and at the molecular levels) may increase with NAFLD severity.

Tools for measuring DNL

Direct measurement of DNL

Mass isotopomer distribution analysis (MIDA) is a gold-standard technique used for the in vivo measurement of DNL by labeling the newly-made FAs (197).

Commonly-used stable isotopes for labeling are deuterated water (d_2O) and ^{13}C -acetate (198, 199). Others have also used ^{13}C -glucose for measuring DNL (200, 201), however, this would mean that the label would have to travel a longer distance to reach FASN for its incorporation into FA (i.e., down glycolysis and into and out of the TCA cycle). Further, because isotope can be lost in other metabolic pathways, a larger amount of isotope would be required making the use of ^{13}C -glucose or ^{13}C -fructose less cost-effective. The rationale for using isotopes closer to the FA synthesis pathway (^{13}C -acetate and d_2O) is shown in **figure 1.7**. When ^{13}C -acetate is used, a carbon molecule enters the FA synthesis pathway immediately after coenzyme A (CoA) is added which eventually labels the newly-made FA. When d_2O is used, then the hydrogen

molecule in FA is replaced by a deuterium molecule via NADPH transfer. Given that 1) hydrogen is required for FA synthesis, and 2) body water is in equilibrium with NADPH, when body water is enriched with deuterium, the deuterium is directly utilized in FA synthesis. In either case, a newly-synthesized FA (in this case palmitate) is one molecular weight heavier (mass to charge weights, $m/z=257$) than regularly-synthesized palmitate. This labeled palmitate molecule is abbreviated as M1, as opposed to M0 ($m/z=256$). Further, during FA synthesis, if the stable-isotope was utilized more than once (e.g., due to greater enrichment of precursor pool), then M2, M3, and M4 can be produced (202). Gas chromatography/mass spectrometry (GC/MS) is used for the detection of FAs of different m/z weights (M0, M1, M2, M3, and M4). Knowing the precursor enrichment of d_2O or acetyl-CoA enrichment, DNL can be calculated using a ratio of excess M2/excess M1 (197, 202).

Furthermore, these calculations in the previous literature have been conducted in palmitate isolated from plasma TRL-TAG but rarely measured directly in liver-TAG (197-199, 202). Only one study conducted by Donnelly et al compared total TAG from the liver tissue and VLDL particles simultaneously and reported that the TAG accounted labeled in the liver was reflected in the TAG labeled in the VLDL particle (103). This study had a sample size of only nine subjects, and studies conducted after this seminal paper were based on the assumption that the DNL measured in the liver was reflected in the VLDL particle. However, no

studies to date have replicated this finding by measured DNL isotopically directly in the liver-TAG and compared to the VLDL-TAG DNL in humans.

Indirect calculators

In addition to cost, the complexity associated with the MIDA requires expertise for measuring DNL using stable isotopes. This led many scientists to develop techniques based on physiological principles that are cost-effective, less complicated, to predict DNL efficiently (203-211). These calculations are primarily based on measuring FA composition using gas chromatography (GC). The following indirect indices of DNL have been established in the literature.

Linoleate dilution - This method (LD) was based on a principle that humans do not synthesize long-chain, polyunsaturated FA such as linoleate (18:2). This technique matches the composition of FA in the diet is that found in the adipose tissue (203). Thus, when FAs are synthesized in the liver, producing saturated FA (SFA) like palmitate, the percentage of 18:2 decreases. Because the primary FA synthesized via the DNL is palmitate (16:0), any changes observed in the ratio of 16:0 and 18:2 can be used as a predictor of DNL. Although this method has been well-established and widely used as an indicator of VLDL-TAG DNL (45, 205, 209, 210), no studies have been conducted to compare the isotopic DNL in the liver-TAG with the ratio of 16:0/18:2 in the plasma or VLDL-TAG.

Desaturation index - Once the FAs are synthesized they can be desaturated via SCD-1 enzyme into monounsaturated FAs (MUFAs). Given the previous relationships observed between the isotopic DNL and SCD-1 activity (204, 208), ratios of VLDL-TAG 16:1n7/16:0 and 18:1n9/18:0 have been used as indicators of liver SCD-1 activity (205-207, 209, 210). One study conducted by Rosqvist et al compared the SCD index with isotopic DNL in the VLDL and reported no relationship between them, however, they reported a trending correlation between isotopic DNL and 16:1n7 in a group of individuals with higher DNL (186). Many other studies have utilized the SCD index found its level in VLDL-TAG is positively associated with liver fat (45, 212, 213). Whether these relationships are reflective of SCD-1 activity in the liver and whether they reflect the direct measurement of DNL (isotopically) in the liver is unknown.

Elongation index - The elongation index (EI) has been assessed by calculating the ratio of 18:0/16:0 and 18:1n7/16:1n7 and used to predict enzymatic activity (205, 209-211). A study conducted by Green et al reported the role of elongases enzymes (ELOV) as an indicator of de novo FAs, particularly 16:1n7 and 18:1n9 (211). However, these methods assume the effect of ELOV requires SCD-1 activity for de novo monounsaturated FA (MUFA) synthesis. The desaturation of DNL FAs to make MUFA has been predicted using the EI in the presence of SCD-1 activity. The relationship between synthesized FA and the EI in liver lipids needs further investigation.

Given the literature cited above, the present dissertation project was designed to accomplish the following specific aims.

SPECIFIC AIMS

Specific Aims 1: In liver biopsy samples from bariatric surgery patients compare in vivo labeling of DNL to histologic documentation of disease severity, and hepatic protein and gene expression of key enzymes involved in the DNL pathway.

Specific Aim 2: In liver biopsy samples from bariatric surgery patients, A) measure DNL in liver-CE FA, B) compare cholesterol levels in the liver and plasma to histologic documentation of disease severity, and C) hepatic protein and gene expression of enzymes involved in the cholesterol metabolism pathways.

Exploratory Aim 1: Compare in vivo labeling of DNL in liver biopsy samples to TRL-TAG DNL.

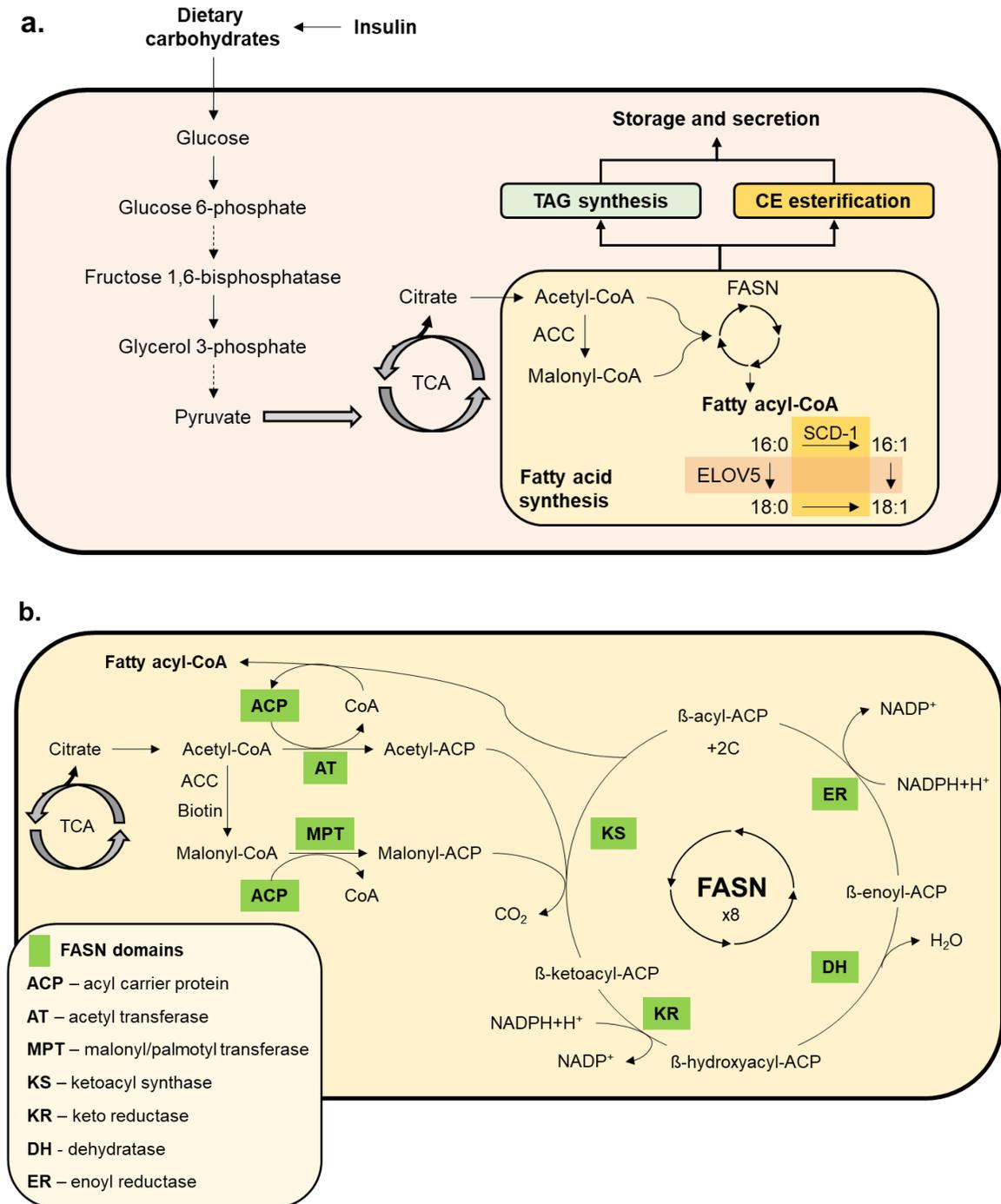
Exploratory Aim 2: Investigate changes in indirect measures of DNL (LD, SCD index, and EI) with A) the disease severity, and compare in vivo labeling of DNL to B) indirect measures in liver biopsy and C) TRL particle.

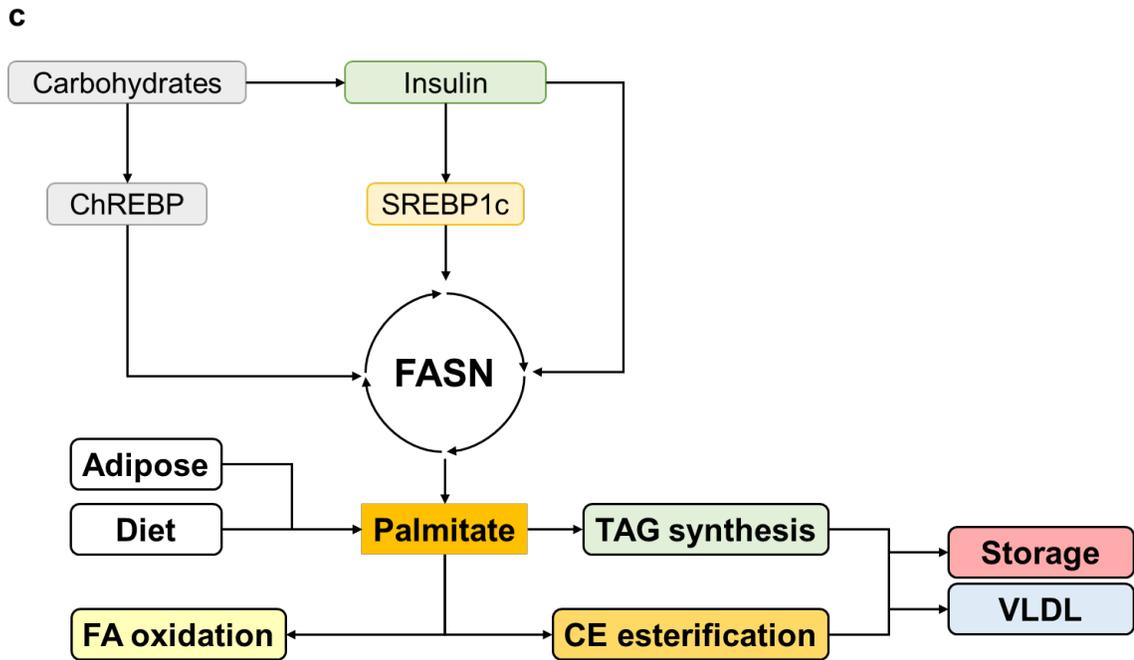
Table 1.1: Summary of gene expression in NAFLD and NASH

Enzymes	NAFLD vs healthy	NASH vs NAFLD	References
<i>Fatty acid uptake</i>			
FATP	↑	↔	(1-6)
FABP	↑	↔	(1-3, 7, 8)
CD36	↑*	↔	(1, 4-8)
<i>DNL key enzymes</i>			
SREBP1	↑	↔	(2, 8-13)
ACC	↑	↔	(1, 2, 8, 14)
FASN	↑	↔	(1, 2, 6, 8, 11, 15)
SCD-1	↑		(1, 6)
ELOV	↑		(7)
<i>TAG metabolism</i>			
DGAT1/2	↑	↔	(6, 13, 14)
MTTP	↑	↓	(1, 2, 13, 16)
<i>VLDL secretion</i>			
apoB100	↑	↓	(1, 2, 13, 16)
<i>FA oxidation</i>			
CPT1	↑		(1, 3)
PPAR-γ	↑	↔	(3, 8)
PPAR-α	↓	↓*↔	(6, 8, 13)
PGC1-α	↓		(6, 17)

Legend: *refers to patients with cirrhosis and HCC. ↑ increased, ↓ decreased, ↔ no difference.

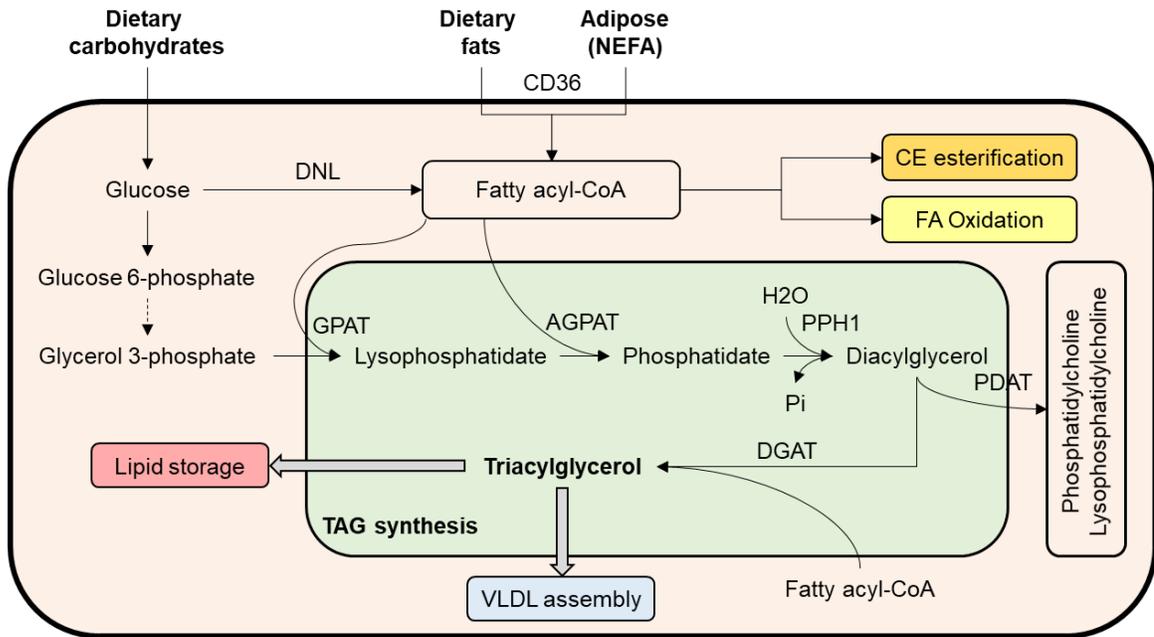
Figure 1.1: Fatty acid synthesis





Legend: Abbreviations:
 TAG - triacylglycerol
 CE - cholesterol ester
 TCA - tricarboxylic cycle
 ACC - acetyl-CoA carboxylase
 FASN - fatty acid synthase
 SCD - stearoyl-CoA desaturase
 ELOV5 - elongases 5
 2C - two carbons

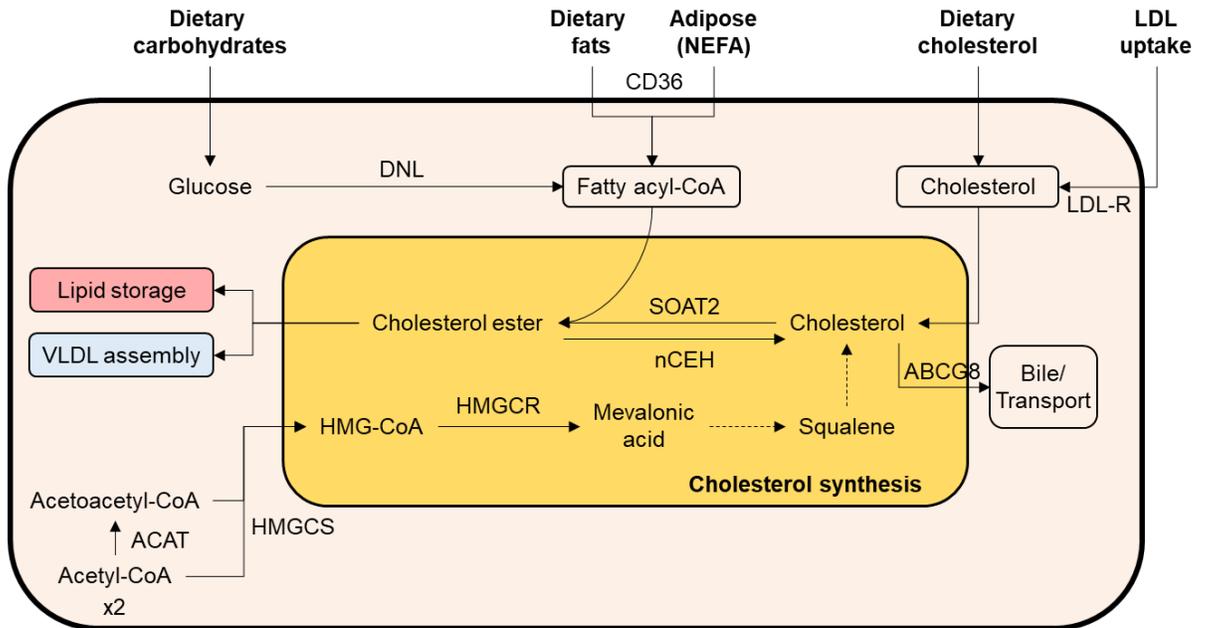
Figure 1.2: Triglycerides synthesis



Legend: Abbreviations:

- NEFA - nonesterified fatty acids
- CD36 - cluster of differentiation 36
- DNL - de novo lipogenesis
- CE - cholesterol ester
- FA - fatty acid
- GPAT - glycerol-3-phosphate
- AGPAT - 1-acylglycerol-3-phosphate-O-acyltransferase
- PPH-1 - phosphohydrolase-1
- PDAT - Phospholipid: diacylglycerol acyltransferase
- Pi - phosphate
- DGAT - diacylglycerol acyltransferase enzyme
- TAG - triacylglycerols
- VLDL - very low-density lipoprotein

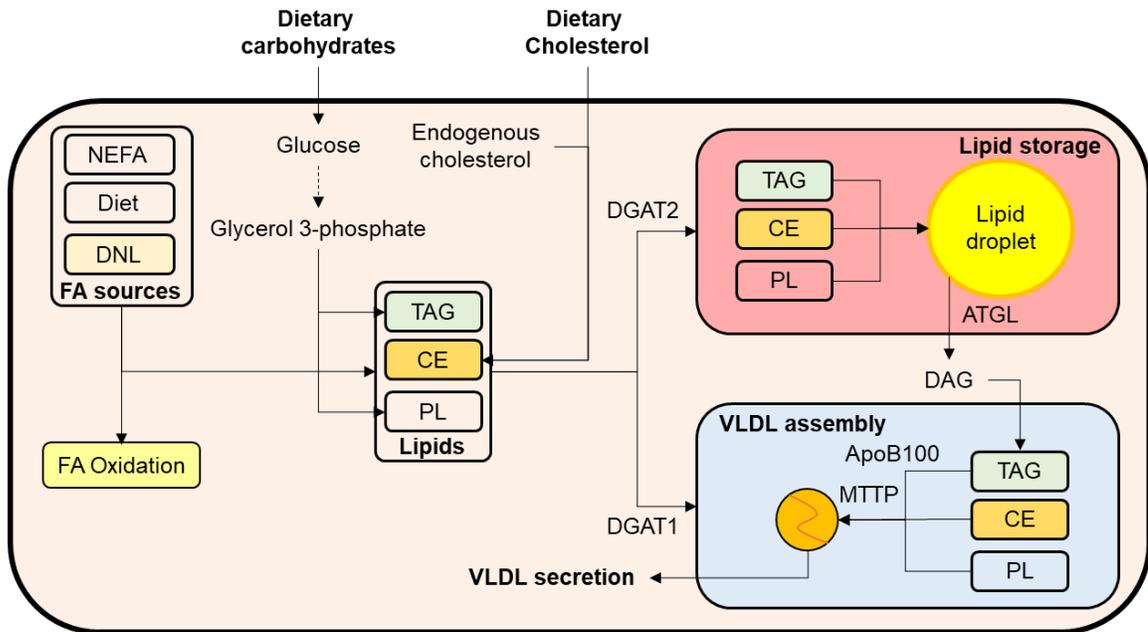
Figure 1.3: Cholesterol synthesis



Legend: Abbreviations:

- NEFA - nonesterified fatty acids
- DNL - de novo lipogenesis
- LDL-R - low-density lipoprotein receptor
- SOAT2 - sterol O-acyltransferase 2
- NCEH1 - neutral cholesterol ester hydrolase 1
- ABCG8 - ATP-binding cassette sub-family G member 8
- HMGCR - β -Hydroxy β -methylglutaryl-CoA reductase
- HMGCS - β -Hydroxy β -methylglutaryl-CoA synthase
- ACAT - acetyl-CoA acetyltransferases

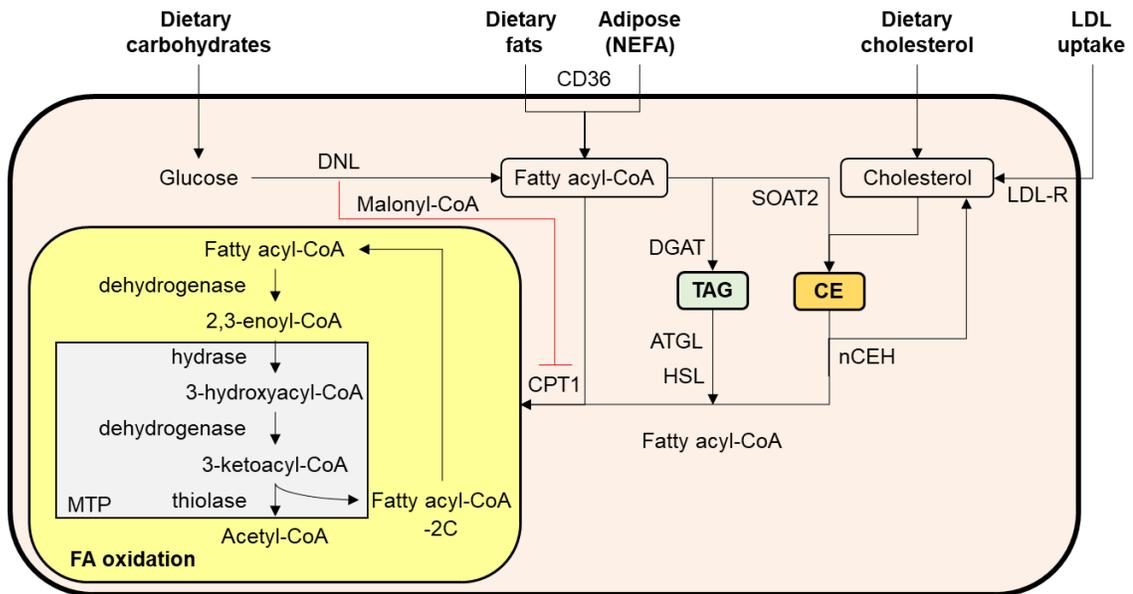
Figure 1.4: Lipid secretion and storage



Legend: Abbreviations:

- NEFA - nonesterified fatty acids
- DNL - de novo lipogenesis
- FA - fatty acid
- TAG - triacylglycerols
- CE - cholesterol esters
- PL - phospholipids
- DGAT - diacylglycerol acyltransferase enzyme
- ATGL - adipose triglyceride lipase
- DAG - diacylglycerols
- VLDL - very low-density lipoprotein
- APOB100 - apolipoprotein B100
- MTP - microsomal triglyceride transfer protein

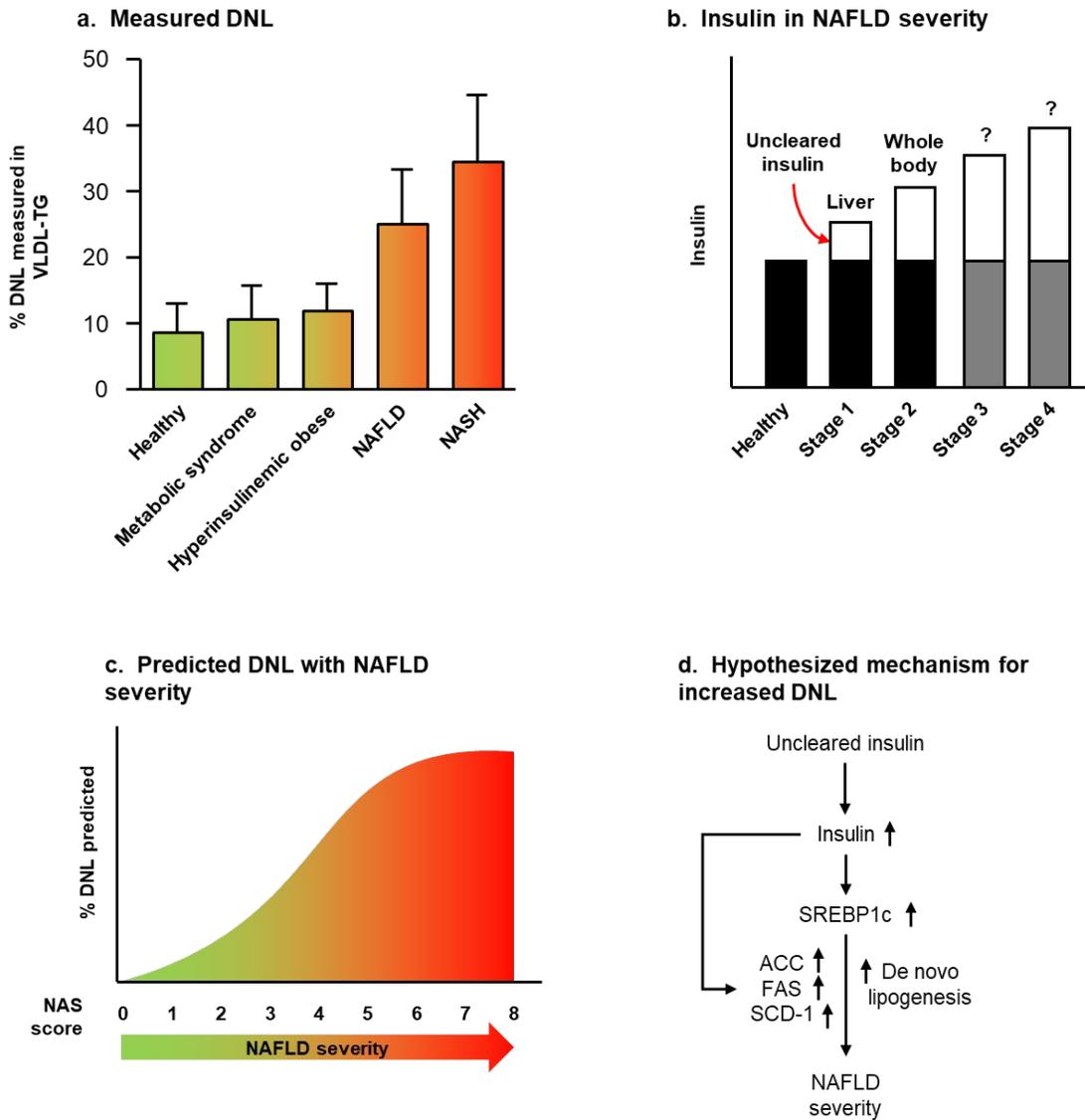
Figure 1.5: Fatty acid oxidation



Legend: Abbreviations:

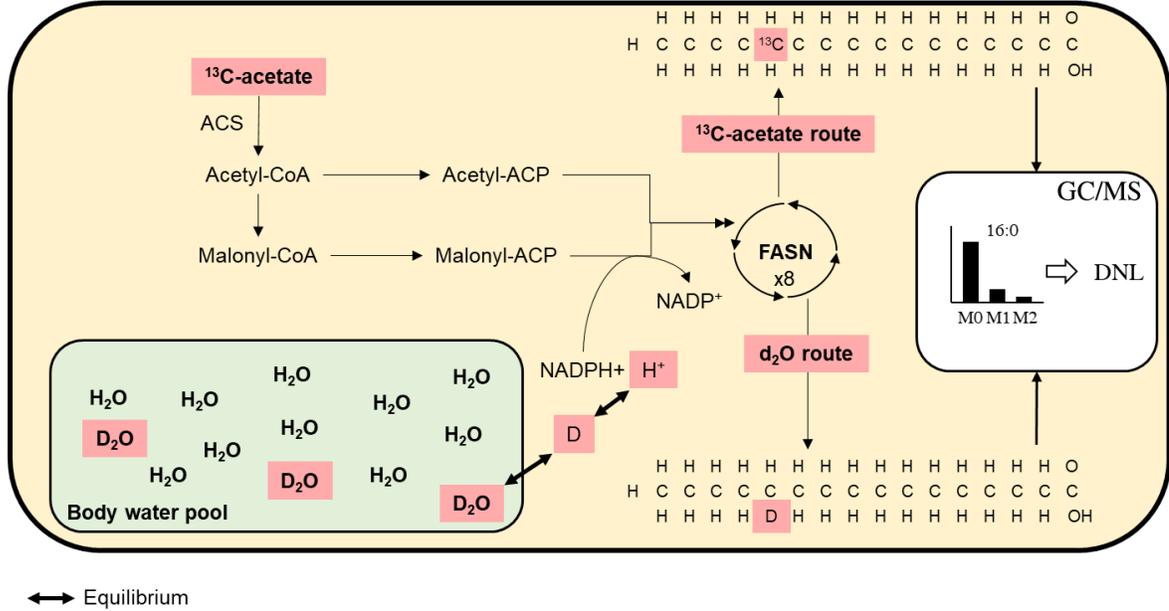
- NEFA - nonesterified fatty acids
- CD36 - cluster of differentiation 36
- DNL - de novo lipogenesis
- SOAT2 - sterol O-acyltransferase 2
- LDL-R - low-density lipoprotein receptor
- DGAT - diacylglycerol acyltransferase enzyme
- TAG - triacylglycerols
- CE - cholesterol esters
- ATGL - Adipose triglyceride lipase
- HSL - hormone-sensitive lipase
- NCEH - neutral cholesterol ester hydrolase
- CPT1 - carnitine palmitoyltransferase 1
- MTP - mitochondrial trifunctional protein
- FA - fatty acid
- 2C - two carbons

Figure 1.6: Measured and predicted DNL



Legend: Data are reported in mean±SD. **1.6a.** DNL data presented in the previous studies was used to calculate an average DNL in different populations. Healthy: n=19 studies, metabolic syndrome: n=8, hyperinsulinemic obese: n=7, NAFLD: n=5, and NASH: n=3. **1.6b.** Predicted insulin concentrations are based on past literature. **1.6c.** Predicted DNL with disease severity based on figure 1.6a. **1.6d.** An insulin-mediated mechanism for driving DNL in NAFLD progression.

Figure 1.7: Labeling of the DNL pathway



REFERENCES

1. Angulo, P. (2002). Nonalcoholic fatty liver disease. *N Engl J Med*, 346(16), 1221-1231. doi:10.1056/NEJMra011775. **PMID: 11961152.**
2. Marchesini, G., Brizi, M., Bianchi, G., Tomassetti, S., Bugianesi, E., Lenzi, M., McCullough, A. J., Natale, S., Forlani, G., & Melchionda, N. (2001). Nonalcoholic fatty liver disease: a feature of the metabolic syndrome. *Diabetes*, 50(8), 1844-1850. doi:10.2337/diabetes.50.8.1844. **PMID: 11473047.**
3. Matteoni, C. A., Younossi, Z. M., Gramlich, T., Boparai, N., Liu, Y. C., & McCullough, A. J. (1999). Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. *Gastroenterology*, 116(6), 1413-1419. doi:10.1016/s0016-5085(99)70506-8. **PMID: 10348825.**
4. Rinella, M. E. (2015). Nonalcoholic fatty liver disease: a systematic review. *JAMA*, 313(22), 2263-2273. doi:10.1001/jama.2015.5370. **PMID: 26057287.**
5. Chalasani, N., Younossi, Z., Lavine, J. E., Diehl, A. M., Brunt, E. M., Cusi, K., Charlton, M., & Sanyal, A. J. (2012). The diagnosis and management of non-alcoholic fatty liver disease: practice Guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. *Hepatology*, 55(6), 2005-2023. doi:10.1002/hep.25762. **PMID: 22488764**
6. Cobbina, E., & Akhlaghi, F. (2017). Non-alcoholic fatty liver disease (NAFLD) - pathogenesis, classification, and effect on drug metabolizing enzymes and transporters. *Drug Metab Rev*, 49(2), 197-211. doi:10.1080/03602532.2017.1293683. **PMC5576152.**
7. NICE-Guidelines. (2016). Diagnosis of NAFLD. In *Non-alcoholic fatty liver disease: Assessment and management*. London: National Guideline Centre (UK).
8. Farrell, G. C., & Larter, C. Z. (2006). Nonalcoholic fatty liver disease: from steatosis to cirrhosis. *Hepatology*, 43(2 Suppl 1), S99-S112. doi:10.1002/hep.20973. **PMID: 16447287.**
9. Sanyal, A. J., & American Gastroenterological, A. (2002). AGA technical review on nonalcoholic fatty liver disease. *Gastroenterology*, 123(5), 1705-1725. doi:10.1053/gast.2002.36572. **PMID: 12404245.**
10. Berlanga, A., Guiu-Jurado, E., Porras, J. A., & Auguet, T. (2014). Molecular pathways in non-alcoholic fatty liver disease. *Clin Exp Gastroenterol*, 7, 221-239. doi:10.2147/CEG.S62831. **PMC4094580.**
11. Ipsen, D. H., Lykkesfeldt, J., & Tveden-Nyborg, P. (2018). Molecular mechanisms of hepatic lipid accumulation in non-alcoholic fatty liver disease. *Cell Mol Life Sci*, 75(18), 3313-3327. doi:10.1007/s00018-018-2860-6. **PMC6105174.**
12. Fabbrini, E., Sullivan, S., & Klein, S. (2010). Obesity and nonalcoholic fatty liver disease: biochemical, metabolic, and clinical implications. *Hepatology*, 51(2), 679-689. doi:10.1002/hep.23280. **PMC3575093.**

13. Lewis, G. F., Carpentier, A., Adeli, K., & Giacca, A. (2002). Disordered fat storage and mobilization in the pathogenesis of insulin resistance and type 2 diabetes. *Endocr Rev*, *23*(2), 201-229. doi:10.1210/edrv.23.2.0461. **PMID: 11943743.**
14. Musso, G., Gambino, R., Cassader, M., & Pagano, G. (2011). Meta-analysis: natural history of non-alcoholic fatty liver disease (NAFLD) and diagnostic accuracy of non-invasive tests for liver disease severity. *Ann Med*, *43*(8), 617-649. doi:10.3109/07853890.2010.518623. **PMID: 21039302**
15. Conus, F., Rabasa-Lhoret, R., & Peronnet, F. (2007). Characteristics of metabolically obese normal-weight (MONW) subjects. *Appl Physiol Nutr Metab*, *32*(1), 4-12. doi:10.1139/H07-926. **PMID: 17332780**
16. Lopez-Velazquez, J. A., Silva-Vidal, K. V., Ponciano-Rodriguez, G., Chavez-Tapia, N. C., Arrese, M., Uribe, M., & Mendez-Sanchez, N. (2014). The prevalence of nonalcoholic fatty liver disease in the Americas. *Ann Hepatol*, *13*(2), 166-178. **PMID: 24552858.**
17. Bugianesi, E., Gastaldelli, A., Vanni, E., Gambino, R., Cassader, M., Baldi, S., Ponti, V., Pagano, G., Ferrannini, E., & Rizzetto, M. (2005). Insulin resistance in non-diabetic patients with non-alcoholic fatty liver disease: sites and mechanisms. *Diabetologia*, *48*(4), 634-642. doi:10.1007/s00125-005-1682-x. **PMID: 15747110.**
18. Younossi, Z., Stepanova, M., Ong, J. P., Jacobson, I. M., Bugianesi, E., Duseja, A., Eguchi, Y., Wong, V. W., Negro, F., Yilmaz, Y., Romero-Gomez, M., George, J., Ahmed, A., Wong, R., Younossi, I., Ziayee, M., Afendy, A., & Global Nonalcoholic Steatohepatitis, C. (2019). Nonalcoholic steatohepatitis is the fastest growing cause of hepatocellular carcinoma in liver transplant candidates. *Clin Gastroenterol Hepatol*, *17*(4), 748-755 e743. doi:10.1016/j.cgh.2018.05.057. **PMID: 29908364.**
19. Younossi, Z. M., Koenig, A. B., Abdelatif, D., Fazel, Y., Henry, L., & Wymer, M. (2016). Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology*, *64*(1), 73-84. doi:10.1002/hep.28431. **PMID: 26707365**
20. Browning, J. D., Szczepaniak, L. S., Dobbins, R., Nuremberg, P., Horton, J. D., Cohen, J. C., Grundy, S. M., & Hobbs, H. H. (2004). Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology*, *40*(6), 1387-1395. doi:10.1002/hep.20466. **PMID: 15565570**
21. Vos, B., Moreno, C., Nagy, N., Fery, F., Cnop, M., Vereerstraeten, P., Deviere, J., & Adler, M. (2011). Lean non-alcoholic fatty liver disease (Lean-NAFLD): a major cause of cryptogenic liver disease. *Acta Gastroenterol Belg*, *74*(3), 389-394. **PMID: 22103042.**
22. Younossi, Z. M., Stepanova, M., Negro, F., Hallaji, S., Younossi, Y., Lam, B., & Srishord, M. (2012). Nonalcoholic fatty liver disease in lean individuals in the United States. *Medicine (Baltimore)*, *91*(6), 319-327. doi:10.1097/MD.0b013e3182779d49. **PMID: 23117851**

23. Younossi, Z. M., Otgonsuren, M., Venkatesan, C., & Mishra, A. (2013). In patients with non-alcoholic fatty liver disease, metabolically abnormal individuals are at a higher risk for mortality while metabolically normal individuals are not. *Metabolism*, 62(3), 352-360. doi:10.1016/j.metabol.2012.08.005. **PMID: 22999011**
24. Diehl, A. M., & Day, C. (2017). Cause, pathogenesis, and treatment of nonalcoholic steatohepatitis. *N Engl J Med*, 377(21), 2063-2072. doi:10.1056/NEJMra1503519. **PMID: 29166236**
25. Rich, N. E., Oji, S., Mufti, A. R., Browning, J. D., Parikh, N. D., Odewole, M., Mayo, H., & Singal, A. G. (2018). Racial and ethnic disparities in nonalcoholic fatty liver disease prevalence, severity, and outcomes in the United States: A systematic review and meta-analysis. *Clin Gastroenterol Hepatol*, 16(2), 198-210 e192. doi:10.1016/j.cgh.2017.09.041. **PMC5794571**.
26. Carroll, R. G., Zaslona, Z., Galvan-Pena, S., Koppe, E. L., Sevin, D. C., Angiari, S., Triantafilou, M., Triantafilou, K., Modis, L. K., & O'Neill, L. A. (2018). An unexpected link between fatty acid synthase and cholesterol synthesis in proinflammatory macrophage activation. *J Biol Chem*, 293(15), 5509-5521. doi:10.1074/jbc.RA118.001921. **PMC5900750**.
27. Hashimoto, E., Taniai, M., & Tokushige, K. (2013). Characteristics and diagnosis of NAFLD/NASH. *J Gastroenterol Hepatol*, 28 Suppl 4, 64-70. doi:10.1111/jgh.12271. **PMID: 24251707**.
28. Wree, A., Broderick, L., Canbay, A., Hoffman, H. M., & Feldstein, A. E. (2013). From NAFLD to NASH to cirrhosis-new insights into disease mechanisms. *Nat Rev Gastroenterol Hepatol*, 10(11), 627-636. doi:10.1038/nrgastro.2013.149. **PMID: 23958599**.
29. Schuster, S., Cabrera, D., Arrese, M., & Feldstein, A. E. (2018). Triggering and resolution of inflammation in NASH. *Nat Rev Gastroenterol Hepatol*, 15(6), 349-364. doi:10.1038/s41575-018-0009-6. **PMID: 29740166**.
30. Blencowe, M., Karunanayake, T., Wier, J., Hsu, N., & Yang, X. (2019). Network modeling approaches and applications to unravelling non-alcoholic fatty liver disease. *Genes (Basel)*, 10(12). doi:10.3390/genes10120966. **PMC6947017**.
31. Spengler, E. K., & Loomba, R. (2015). Recommendations for diagnosis, referral for liver biopsy, and treatment of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. *Mayo Clin Proc*, 90(9), 1233-1246. doi:10.1016/j.mayocp.2015.06.013. **PMC4567478**.
32. Tsochatzis, E. A., Bosch, J., & Burroughs, A. K. (2014). Liver cirrhosis. *Lancet*, 383(9930), 1749-1761. doi:10.1016/S0140-6736(14)60121-5. **PMID: 24480518**.
33. Anstee, Q. M., Targher, G., & Day, C. P. (2013). Progression of NAFLD to diabetes mellitus, cardiovascular disease or cirrhosis. *Nat Rev Gastroenterol Hepatol*, 10(6), 330-344. doi:10.1038/nrgastro.2013.41. **PMID: 23507799**.
34. Ratziu, V., Bellentani, S., Cortez-Pinto, H., Day, C., & Marchesini, G. (2010). A position statement on NAFLD/NASH based on the EASL 2009

- special conference. *J Hepatol*, 53(2), 372-384.
doi:10.1016/j.jhep.2010.04.008. **PMID: 20494470**
35. Younossi, Z., Anstee, Q. M., Marietti, M., Hardy, T., Henry, L., Eslam, M., George, J., & Bugianesi, E. (2018). Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. *Nat Rev Gastroenterol Hepatol*, 15(1), 11-20. doi:10.1038/nrgastro.2017.109. **PMID: 28930295**
 36. Fazel, Y., Koenig, A. B., Sayiner, M., Goodman, Z. D., & Younossi, Z. M. (2016). Epidemiology and natural history of non-alcoholic fatty liver disease. *Metabolism*, 65(8), 1017-1025.
doi:10.1016/j.metabol.2016.01.012. **PMID: 26997539**
 37. Farrell, G. C., Chitturi, S., Lau, G. K., Sollano, J. D., & Asia-Pacific Working Party on, N. (2007). Guidelines for the assessment and management of non-alcoholic fatty liver disease in the Asia-Pacific region: executive summary. *J Gastroenterol Hepatol*, 22(6), 775-777. doi:10.1111/j.1440-1746.2007.05002.x. **PMID: 17565629**.
 38. Feijo, S. G., Lima, J. M., Oliveira, M. A., Patrocinio, R. M., Moura-Junior, L. G., Campos, A. B., Lima, J. W., & Braga, L. L. (2013). The spectrum of non alcoholic fatty liver disease in morbidly obese patients: prevalence and associate risk factors. *Acta Cir Bras*, 28(11), 788-793. **PMID: 24316747**.
 39. Neuschwander-Tetri, B. A., & Caldwell, S. H. (2003). Nonalcoholic steatohepatitis: summary of an AASLD Single Topic Conference. *Hepatology*, 37(5), 1202-1219. doi:10.1053/jhep.2003.50193. **PMID: 12717402**.
 40. Sherif, Z. A., Saeed, A., Ghavimi, S., Nouraie, S. M., Laiyemo, A. O., Brim, H., & Ashktorab, H. (2016). Global epidemiology of nonalcoholic fatty liver disease and perspectives on US minority populations. *Dig Dis Sci*, 61(5), 1214-1225. doi:10.1007/s10620-016-4143-0. **PMC4838529**.
 41. Kabbany, M. N., Conjeevaram Selvakumar, P. K., Watt, K., Lopez, R., Akras, Z., Zein, N., Carey, W., & Alkhoury, N. (2017). Prevalence of nonalcoholic steatohepatitis-associated cirrhosis in the United States: An analysis of National Health and Nutrition Examination Survey data. *Am J Gastroenterol*, 112(4), 581-587. doi:10.1038/ajg.2017.5. **PMID: 28195177**
 42. Ekstedt, M., Franzen, L. E., Mathiesen, U. L., Thorelius, L., Holmqvist, M., Bodemar, G., & Kechagias, S. (2006). Long-term follow-up of patients with NAFLD and elevated liver enzymes. *Hepatology*, 44(4), 865-873.
doi:10.1002/hep.21327. **PMID: 17006923**
 43. Ludwig, J., Viggiano, T. R., McGill, D. B., & Oh, B. J. (1980). Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. *Mayo Clin Proc*, 55(7), 434-438. **PMID: 7382552**.
 44. Lambert, J. E., Ramos-Roman, M. A., Browning, J. D., & Parks, E. J. (2014). Increased de novo lipogenesis is a distinct characteristic of individuals with nonalcoholic fatty liver disease. *Gastroenterology*, 146(3), 726-735. doi:10.1053/j.gastro.2013.11.049. **PMC6276362**.

45. Lee, J. J., Lambert, J. E., Hovhannisyan, Y., Ramos-Roman, M. A., Trombold, J. R., Wagner, D. A., & Parks, E. J. (2015). Palmitoleic acid is elevated in fatty liver disease and reflects hepatic lipogenesis. *Am J Clin Nutr*, *101*(1), 34-43. doi:10.3945/ajcn.114.092262. **PMC4266891**.
46. Lawitz, E. J., Coste, A., Poordad, F., Alkhouri, N., Loo, N., McColgan, B. J., Tarrant, J. M., Nguyen, T., Han, L., Chung, C., Ray, A. S., McHutchison, J. G., Subramanian, G. M., Myers, R. P., Middleton, M. S., Sirlin, C., Loomba, R., Nyangau, E., Fitch, M., Li, K., & Hellerstein, M. (2018). Acetyl-CoA carboxylase inhibitor GS-0976 for 12 weeks reduces hepatic de novo lipogenesis and steatosis in patients with nonalcoholic steatohepatitis. *Clin Gastroenterol Hepatol*, *16*(12), 1983-1991 e1983. doi:10.1016/j.cgh.2018.04.042. **PMID: 29705265**
47. Loomba, R., Kayali, Z., Nouredin, M., Ruane, P., Lawitz, E. J., Bennett, M., Wang, L., Harting, E., Tarrant, J. M., McColgan, B. J., Chung, C., Ray, A. S., Subramanian, G. M., Myers, R. P., Middleton, M. S., Lai, M., Charlton, M., & Harrison, S. A. (2018). GS-0976 reduces hepatic steatosis and fibrosis markers in patients with nonalcoholic fatty liver disease. *Gastroenterology*, *155*(5), 1463-1473 e1466. doi:10.1053/j.gastro.2018.07.027. **PMC6318218**.
48. Lawitz, E., Li, K., Tarrant, J., Vimal, M., Xu, R., Song, Q., Schall, R. E. A., McColgan, B. J., Djedjos, C. S., Ray, A. S., Myers, R. P., Hellerstein, M. K., & Loomba, R. (2017). Hepatic de novo lipogenesis is elevated in patients with NASH independent of disease severity. *Hepatology*, *66*(1 (Suppl)), 1173A. doi:doi: 10.1002/hep.29500. **PMID: 28965360**
49. Kim, C. W., Addy, C., Kusunoki, J., Anderson, N. N., Deja, S., Fu, X., Burgess, S. C., Li, C., Ruddy, M., Chakravarthy, M., Previs, S., Milstein, S., Fitzgerald, K., Kelley, D. E., & Horton, J. D. (2017). Acetyl CoA carboxylase inhibition reduces hepatic steatosis but elevates plasma triglycerides in mice and humans: A bedside to bench investigation. *Cell Metab*, *26*(2), 394-406 e396. doi:10.1016/j.cmet.2017.07.009. **PMC5603267**.
50. Parks, E. J., Manrique, C. M., Syed-Abdul, M. M., Gaballah, A. H., Hammoud, G. M., Buckley, D., Duke, G., McCulloch, W., & Kemble, G. (2017). Pharmacologic inhibition of FASN reverses diet-induced steatohepatitis in mice and inhibits lipogenesis in humans. *Hepatology*, *66*(1 (Suppl)), 1045A. doi:10.1002/hep.29500. **PMID: 28965360**.
51. Syed-Abdul, M. M., Parks, E. J., Gaballah, A. H., Bingham, K., Hammoud, G. M., Kemble, G., Buckley, D., McCulloch, W., & Manrique-Acevedo, C. (2020). Fatty acid synthase inhibitor TVB-2640 reduces hepatic de novo lipogenesis in males with metabolic abnormalities. *Hepatology*, *72*(1), 103-118. doi:10.1002/hep.31000. **PMID: 31630414**.
52. Fabbrini, E., Mohammed, B. S., Magkos, F., Korenblat, K. M., Patterson, B. W., & Klein, S. (2008). Alterations in adipose tissue and hepatic lipid kinetics in obese men and women with nonalcoholic fatty liver disease. *Gastroenterology*, *134*(2), 424-431. doi:10.1053/j.gastro.2007.11.038. **PMC2705923**.

53. Fabbrini, E., deHaseth, D., Deivanayagam, S., Mohammed, B. S., Vitola, B. E., & Klein, S. (2009). Alterations in fatty acid kinetics in obese adolescents with increased intrahepatic triglyceride content. *Obesity (Silver Spring)*, 17(1), 25-29. doi:10.1038/oby.2008.494. **PMC2649753**.
54. Fabbrini, E., Mohammed, B. S., Korenblat, K. M., Magkos, F., McCrea, J., Patterson, B. W., & Klein, S. (2010). Effect of fenofibrate and niacin on intrahepatic triglyceride content, very low-density lipoprotein kinetics, and insulin action in obese subjects with nonalcoholic fatty liver disease. *J Clin Endocrinol Metab*, 95(6), 2727-2735. doi:10.1210/jc.2009-2622. **PMC2902076**.
55. Calzadilla Bertot, L., & Adams, L. A. (2016). The natural course of non-alcoholic fatty liver disease. *Int J Mol Sci*, 17(5). doi:10.3390/ijms17050774. **PMC4881593**.
56. Browning, J. D., & Horton, J. D. (2004). Molecular mediators of hepatic steatosis and liver injury. *J Clin Invest*, 114(2), 147-152. doi:10.1172/JCI22422. **PMC449757**.
57. Malhi, H., & Gores, G. J. (2008). Molecular mechanisms of lipotoxicity in nonalcoholic fatty liver disease. *Semin Liver Dis*, 28(4), 360-369. doi:10.1055/s-0028-1091980. **PMC2908270**.
58. Chakravarthy, M. V., Waddell, T., Banerjee, R., & Guess, N. (2020). Nutrition and nonalcoholic fatty liver disease: Current perspectives. *Gastroenterol Clin North Am*, 49(1), 63-94. doi:10.1016/j.gtc.2019.09.003. **PMID: 32033765**.
59. Boogaerts, J. R., Malone-McNeal, M., Archambault-Schexnayder, J., & Davis, R. A. (1984). Dietary carbohydrate induces lipogenesis and very-low-density lipoprotein synthesis. *Am J Physiol*, 246(1 Pt 1), E77-83. doi:10.1152/ajpendo.1984.246.1.E77. **PMID: 6696065**.
60. Parks, E. J. (2001). Effect of dietary carbohydrate on triglyceride metabolism in humans. *J Nutr*, 131(10), 2772S-2774S. doi:10.1093/jn/131.10.2772S. **PMID: 11584104**.
61. McDevitt, R. M., Bott, S. J., Harding, M., Coward, W. A., Bluck, L. J., & Prentice, A. M. (2001). De novo lipogenesis during controlled overfeeding with sucrose or glucose in lean and obese women. *Am J Clin Nutr*, 74(6), 737-746. doi:10.1093/ajcn/74.6.737. **PMID: 11722954**
62. Softic, S., Cohen, D. E., & Kahn, C. R. (2016). Role of dietary fructose and hepatic de novo lipogenesis in fatty liver disease. *Dig Dis Sci*, 61(5), 1282-1293. doi:10.1007/s10620-016-4054-0. **PMC4838515**.
63. Charidemou, E., Ashmore, T., Li, X., McNally, B. D., West, J. A., Liggi, S., Harvey, M., Orford, E., & Griffin, J. L. (2019). A randomized 3-way crossover study indicates that high-protein feeding induces de novo lipogenesis in healthy humans. *JCI Insight*, 4(12). doi:10.1172/jci.insight.124819. **PMC6629161**.
64. Parks, E., Yki-Jarvinen, H., & Hawkins, M. (2017). Out of the frying pan: dietary saturated fat influences nonalcoholic fatty liver disease. *J Clin Invest*, 127(2), 454-456. doi:10.1172/JCI92407. **PMC5272176**.

65. Hernandez, E. A., Kahl, S., Seelig, A., Begovatz, P., Irmeler, M., Kupriyanova, Y., Nowotny, B., Nowotny, P., Herder, C., Barosa, C., Carvalho, F., Rozman, J., Neschen, S., Jones, J. G., Beckers, J., de Angelis, M. H., & Roden, M. (2017). Acute dietary fat intake initiates alterations in energy metabolism and insulin resistance. *J Clin Invest*, *127*(2), 695-708. doi:10.1172/JCI89444. **PMC5272194**.
66. Rosqvist, F., Kullberg, J., Stahlman, M., Cedernaes, J., Heurling, K., Johansson, H. E., Iggman, D., Wilking, H., Larsson, A., Eriksson, O., Johansson, L., Straniero, S., Rudling, M., Antoni, G., Lubberink, M., Orholm-Melander, M., Boren, J., Ahlstrom, H., & Riserus, U. (2019). Overeating saturated fat promotes fatty liver and ceramides compared with polyunsaturated fat: A randomized trial. *J Clin Endocrinol Metab*, *104*(12), 6207-6219. doi:10.1210/jc.2019-00160. **PMC6839433**.
67. Velazquez, K. T., Enos, R. T., Bader, J. E., Sougiannis, A. T., Carson, M. S., Chatzistamou, I., Carson, J. A., Nagarkatti, P. S., Nagarkatti, M., & Murphy, E. A. (2019). Prolonged high-fat-diet feeding promotes non-alcoholic fatty liver disease and alters gut microbiota in mice. *World J Hepatol*, *11*(8), 619-637. doi:10.4254/wjh.v11.i8.619. **PMC6717713**.
68. Basaranoglu, M., Basaranoglu, G., & Bugianesi, E. (2015). Carbohydrate intake and nonalcoholic fatty liver disease: fructose as a weapon of mass destruction. *Hepatobiliary Surg Nutr*, *4*(2), 109-116. doi:10.3978/j.issn.2304-3881.2014.11.05. **PMC4405421**.
69. Lim, J. S., Mietus-Snyder, M., Valente, A., Schwarz, J. M., & Lustig, R. H. (2010). The role of fructose in the pathogenesis of NAFLD and the metabolic syndrome. *Nat Rev Gastroenterol Hepatol*, *7*(5), 251-264. doi:10.1038/nrgastro.2010.41. **PMID: 20368739**.
70. Min, H. K., Kapoor, A., Fuchs, M., Mirshahi, F., Zhou, H., Maher, J., Kellum, J., Warnick, R., Contos, M. J., & Sanyal, A. J. (2012). Increased hepatic synthesis and dysregulation of cholesterol metabolism is associated with the severity of nonalcoholic fatty liver disease. *Cell Metab*, *15*(5), 665-674. doi:10.1016/j.cmet.2012.04.004. **PMC3361911**.
71. Puri, P., Baillie, R. A., Wiest, M. M., Mirshahi, F., Choudhury, J., Cheung, O., Sargeant, C., Contos, M. J., & Sanyal, A. J. (2007). A lipidomic analysis of nonalcoholic fatty liver disease. *Hepatology*, *46*(4), 1081-1090. doi:10.1002/hep.21763. **PMID: 17654743**
72. Ferre, P., & Foufelle, F. (2010). Hepatic steatosis: a role for de novo lipogenesis and the transcription factor SREBP-1c. *Diabetes Obes Metab*, *12 Suppl 2*, 83-92. doi:10.1111/j.1463-1326.2010.01275.x. **PMID: 21029304**.
73. Jump, D. B., Tripathy, S., & Depner, C. M. (2013). Fatty acid-regulated transcription factors in the liver. *Annu Rev Nutr*, *33*, 249-269. doi:10.1146/annurev-nutr-071812-161139. **PMC3940310**.
74. Grevengoed, T. J., Klett, E. L., & Coleman, R. A. (2014). Acyl-CoA metabolism and partitioning. *Annu Rev Nutr*, *34*, 1-30. doi:10.1146/annurev-nutr-071813-105541. **PMC5881898**.

75. Mao, J., DeMayo, F. J., Li, H., Abu-Elheiga, L., Gu, Z., Shaikenov, T. E., Kordari, P., Chirala, S. S., Heird, W. C., & Wakil, S. J. (2006). Liver-specific deletion of acetyl-CoA carboxylase 1 reduces hepatic triglyceride accumulation without affecting glucose homeostasis. *Proc Natl Acad Sci U S A*, *103*(22), 8552-8557. doi:10.1073/pnas.0603115103. **PMC1570106**.
76. Savage, D. B., Choi, C. S., Samuel, V. T., Liu, Z. X., Zhang, D., Wang, A., Zhang, X. M., Cline, G. W., Yu, X. X., Geisler, J. G., Bhanot, S., Monia, B. P., & Shulman, G. I. (2006). Reversal of diet-induced hepatic steatosis and hepatic insulin resistance by antisense oligonucleotide inhibitors of acetyl-CoA carboxylases 1 and 2. *J Clin Invest*, *116*(3), 817-824. doi:10.1172/JCI27300. **PMC1366503**.
77. Lee, A. K., Kyriakou, T., Weston, A. J., & O'Dell, S. D. (2010). Functional single-nucleotide polymorphism in acetyl-CoA carboxylase ACACB gene promoter. *DNA Cell Biol*, *29*(12), 703-712. doi:10.1089/dna.2010.1078. **PMID: 20799892**
78. Chakravarthy, M. V., Pan, Z., Zhu, Y., Tordjman, K., Schneider, J. G., Coleman, T., Turk, J., & Semenkovich, C. F. (2005). "New" hepatic fat activates PPARalpha to maintain glucose, lipid, and cholesterol homeostasis. *Cell Metab*, *1*(5), 309-322. doi:10.1016/j.cmet.2005.04.002. **PMID: 16054078**
79. Wu, M., Singh, S. B., Wang, J., Chung, C. C., Salituro, G., Karanam, B. V., Lee, S. H., Powles, M., Ellsworth, K. P., Lassman, M. E., Miller, C., Myers, R. W., Tota, M. R., Zhang, B. B., & Li, C. (2011). Antidiabetic and antisteatotic effects of the selective fatty acid synthase (FAS) inhibitor platensimycin in mouse models of diabetes. *Proc Natl Acad Sci U S A*, *108*(13), 5378-5383. doi:10.1073/pnas.1002588108. **PMC3069196**.
80. Ntambi, J. M., Miyazaki, M., Stoehr, J. P., Lan, H., Kendzioriski, C. M., Yandell, B. S., Song, Y., Cohen, P., Friedman, J. M., & Attie, A. D. (2002). Loss of stearoyl-CoA desaturase-1 function protects mice against adiposity. *Proc Natl Acad Sci U S A*, *99*(17), 11482-11486. doi:10.1073/pnas.132384699. **PMC123282**.
81. Cohen, P., Miyazaki, M., Socci, N. D., Hagge-Greenberg, A., Liedtke, W., Soukas, A. A., Sharma, R., Hudgins, L. C., Ntambi, J. M., & Friedman, J. M. (2002). Role for stearoyl-CoA desaturase-1 in leptin-mediated weight loss. *Science*, *297*(5579), 240-243. doi:10.1126/science.1071527. **PMID: 12114623**.
82. Jiang, G., Li, Z., Liu, F., Ellsworth, K., Dallas-Yang, Q., Wu, M., Ronan, J., Esau, C., Murphy, C., Szalkowski, D., Bergeron, R., Doebber, T., & Zhang, B. B. (2005). Prevention of obesity in mice by antisense oligonucleotide inhibitors of stearoyl-CoA desaturase-1. *J Clin Invest*, *115*(4), 1030-1038. doi:10.1172/JCI23962. **PMC1062893**.
83. Dif, N., Euthine, V., Gonnet, E., Laville, M., Vidal, H., & Lefai, E. (2006). Insulin activates human sterol-regulatory-element-binding protein-1c (SREBP-1c) promoter through SRE motifs. *Biochem J*, *400*(1), 179-188. doi:10.1042/BJ20060499. **PMC1635455**.

84. Matsuzaka, T., & Shimano, H. (2013). Insulin-dependent and -independent regulation of sterol regulatory element-binding protein-1c. *J Diabetes Investig*, 4(5), 411-412. doi:10.1111/jdi.12098. **PMC4025107**.
85. Samuel, V. T., & Shulman, G. I. (2018). Nonalcoholic fatty liver disease as a nexus of metabolic and hepatic diseases. *Cell Metab*, 27(1), 22-41. doi:10.1016/j.cmet.2017.08.002. **PMC5762395**.
86. Abdul-Wahed, A., Guilmeau, S., & Postic, C. (2017). Sweet sixteenth for ChREBP: Established roles and future goals. *Cell Metab*, 26(2), 324-341. doi:10.1016/j.cmet.2017.07.004. **PMID: 28768172**.
87. Dentin, R., Tomas-Cobos, L., Foufelle, F., Leopold, J., Girard, J., Postic, C., & Ferre, P. (2012). Glucose 6-phosphate, rather than xylulose 5-phosphate, is required for the activation of ChREBP in response to glucose in the liver. *J Hepatol*, 56(1), 199-209. doi:10.1016/j.jhep.2011.07.019. **PMID: 21835137**.
88. Ortega-Prieto, P., & Postic, C. (2019). Carbohydrate Sensing Through the Transcription Factor ChREBP. *Front Genet*, 10, 472. doi:10.3389/fgene.2019.00472. **PMC6593282**.
89. Postic, C., & Girard, J. (2008). Contribution of de novo fatty acid synthesis to hepatic steatosis and insulin resistance: lessons from genetically engineered mice. *J Clin Invest*, 118(3), 829-838. doi:10.1172/JCI34275. **PMC2254980**.
90. Benhamed, F., Denechaud, P. D., Lemoine, M., Robichon, C., Moldes, M., Bertrand-Michel, J., Ratziu, V., Serfaty, L., Housset, C., Capeau, J., Girard, J., Guillou, H., & Postic, C. (2012). The lipogenic transcription factor ChREBP dissociates hepatic steatosis from insulin resistance in mice and humans. *J Clin Invest*, 122(6), 2176-2194. doi:10.1172/JCI41636. **PMC3366390**.
91. Chambers, K. T., Chen, Z., Lai, L., Leone, T. C., Towle, H. C., Kralli, A., Crawford, P. A., & Finck, B. N. (2013). PGC-1beta and ChREBP partner to cooperatively regulate hepatic lipogenesis in a glucose concentration-dependent manner. *Mol Metab*, 2(3), 194-204. doi:10.1016/j.molmet.2013.05.001. **PMC3773825**.
92. Uyeda, K., & Repa, J. J. (2006). Carbohydrate response element binding protein, ChREBP, a transcription factor coupling hepatic glucose utilization and lipid synthesis. *Cell Metab*, 4(2), 107-110. doi:10.1016/j.cmet.2006.06.008. **PMID: 16890538**.
93. Iroz, A., Montagner, A., Benhamed, F., Levavasseur, F., Polizzi, A., Anthony, E., Regnier, M., Fouche, E., Lukowicz, C., Cauzac, M., Tournier, E., Do-Cruzeiro, M., Daujat-Chavanieu, M., Gerbal-Chalouin, S., Fauveau, V., Marmier, S., Burnol, A. F., Guilmeau, S., Lippi, Y., Girard, J., Wahli, W., Dentin, R., Guillou, H., & Postic, C. (2017). A specific ChREBP and PPARalpha cross-talk is required for the glucose-mediated FGF21 response. *Cell Rep*, 21(2), 403-416. doi:10.1016/j.celrep.2017.09.065. **PMC5643524**.
94. Baraille, F., Planchais, J., Dentin, R., Guilmeau, S., & Postic, C. (2015). Integration of ChREBP-mediated glucose sensing into whole body

- metabolism. *Physiology (Bethesda)*, 30(6), 428-437. doi:10.1152/physiol.00016.2015. **PMID: 26525342**.
95. Jois, T., Chen, W., Howard, V., Harvey, R., Youngs, K., Thalmann, C., Saha, P., Chan, L., Cowley, M. A., & Sleeman, M. W. (2017). Deletion of hepatic carbohydrate response element binding protein (ChREBP) impairs glucose homeostasis and hepatic insulin sensitivity in mice. *Mol Metab*, 6(11), 1381-1394. doi:10.1016/j.molmet.2017.07.006. **PMID: 285681238**.
 96. Denechaud, P. D., Dentin, R., Girard, J., & Postic, C. (2008). Role of ChREBP in hepatic steatosis and insulin resistance. *FEBS Lett*, 582(1), 68-73. doi:10.1016/j.febslet.2007.07.084. **PMID: 17716660**
 97. Erion, D. M., Popov, V., Hsiao, J. J., Vatner, D., Mitchell, K., Yonemitsu, S., Nagai, Y., Kahn, M., Gillum, M. P., Dong, J., Murray, S. F., Manchem, V. P., Bhanot, S., Cline, G. W., Shulman, G. I., & Samuel, V. T. (2013). The role of the carbohydrate response element-binding protein in male fructose-fed rats. *Endocrinology*, 154(1), 36-44. doi:10.1210/en.2012-1725. **PMID: 23529388**.
 98. Oh, A. R., Sohn, S., Lee, J., Park, J. M., Nam, K. T., Hahm, K. B., Kim, Y. B., Lee, H. J., & Cha, J. Y. (2018). ChREBP deficiency leads to diarrhea-predominant irritable bowel syndrome. *Metabolism*, 85, 286-297. doi:10.1016/j.metabol.2018.04.006. **PMID: 297400734**.
 99. Hall, A. M., & Finck, B. N. (2017). ChREBP refines the hepatic response to fructose to protect the liver from injury. *J Clin Invest*, 127(7), 2533-2535. doi:10.1172/JCI95008. **PMID: 285490753**.
 100. Zhang, D., Tong, X., VanDommelen, K., Gupta, N., Stamper, K., Brady, G. F., Meng, Z., Lin, J., Rui, L., Omary, M. B., & Yin, L. (2017). Lipogenic transcription factor ChREBP mediates fructose-induced metabolic adaptations to prevent hepatotoxicity. *J Clin Invest*, 127(7), 2855-2867. doi:10.1172/JCI89934. **PMID: 285490767**.
 101. Eissing, L., Scherer, T., Todter, K., Knippschild, U., Greve, J. W., Buurman, W. A., Pinnschmidt, H. O., Rensen, S. S., Wolf, A. M., Bartelt, A., Heeren, J., Buettner, C., & Scheja, L. (2013). De novo lipogenesis in human fat and liver is linked to ChREBP-beta and metabolic health. *Nat Commun*, 4, 1528. doi:10.1038/ncomms2537. **PMID: 23740744**.
 102. Haas, J. T., Miao, J., Chanda, D., Wang, Y., Zhao, E., Haas, M. E., Hirschey, M., Vaitheesvaran, B., Farese, R. V., Jr., Kurland, I. J., Graham, M., Crooke, R., Foufelle, F., & Biddinger, S. B. (2012). Hepatic insulin signaling is required for obesity-dependent expression of SREBP-1c mRNA but not for feeding-dependent expression. *Cell Metab*, 15(6), 873-884. doi:10.1016/j.cmet.2012.05.002. **PMID: 223383842**.
 103. Donnelly, K. L., Smith, C. I., Schwarzenberg, S. J., Jessurun, J., Boldt, M. D., & Parks, E. J. (2005). Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J Clin Invest*, 115(5), 1343-1351. doi:10.1172/JCI23621. **PMID: 1587172**.
 104. Barrows, B. R., & Parks, E. J. (2006). Contributions of different fatty acid sources to very low-density lipoprotein-triacylglycerol in the fasted and fed

- states. *J Clin Endocrinol Metab*, 91(4), 1446-1452. doi:10.1210/jc.2005-1709. **PMID: 16449340**.
105. Donnelly, K. L., Margosian, M. R., Sheth, S. S., Lusic, A. J., & Parks, E. J. (2004). Increased lipogenesis and fatty acid reesterification contribute to hepatic triacylglycerol stores in hyperlipidemic Txnip^{-/-} mice. *J Nutr*, 134(6), 1475-1480. doi:10.1093/jn/134.6.1475. **PMID: 15173414**.
 106. Santoro, N., Caprio, S., Pierpont, B., Van Name, M., Savoye, M., & Parks, E. J. (2015). Hepatic De Novo Lipogenesis in Obese Youth Is Modulated by a Common Variant in the GCKR Gene. *J Clin Endocrinol Metab*, 100(8), E1125-1132. doi:10.1210/jc.2015-1587. **PMC4524990**.
 107. Schwarz, J. M., Linfoot, P., Dare, D., & Aghajanian, K. (2003). Hepatic de novo lipogenesis in normoinsulinemic and hyperinsulinemic subjects consuming high-fat, low-carbohydrate and low-fat, high-carbohydrate isoenergetic diets. *Am J Clin Nutr*, 77(1), 43-50. doi:10.1093/ajcn/77.1.43. **PMID: 12499321**.
 108. Stanhope, K. L., Schwarz, J. M., Keim, N. L., Griffen, S. C., Bremer, A. A., Graham, J. L., Hatcher, B., Cox, C. L., Dyachenko, A., Zhang, W., McGahan, J. P., Seibert, A., Krauss, R. M., Chiu, S., Schaefer, E. J., Ai, M., Otokoza, S., Nakajima, K., Nakano, T., Beysen, C., Hellerstein, M. K., Berglund, L., & Havel, P. J. (2009). Consuming fructose-sweetened, not glucose-sweetened, beverages increases visceral adiposity and lipids and decreases insulin sensitivity in overweight/obese humans. *J Clin Invest*, 119(5), 1322-1334. doi:10.1172/JCI37385. **PMC2673878**.
 109. Cohen, J. C., Horton, J. D., & Hobbs, H. H. (2011). Human fatty liver disease: old questions and new insights. *Science*, 332(6037), 1519-1523. doi:10.1126/science.1204265. **PMC3229276**.
 110. Rotman, Y., & Sanyal, A. J. (2017). Current and upcoming pharmacotherapy for non-alcoholic fatty liver disease. *Gut*, 66(1), 180-190. doi:10.1136/gutjnl-2016-312431. **PMID: 27646933**.
 111. Sumida, Y., & Yoneda, M. (2018). Current and future pharmacological therapies for NAFLD/NASH. *J Gastroenterol*, 53(3), 362-376. doi:10.1007/s00535-017-1415-1. **PMC5847174**.
 112. Esler, W. P., & Bence, K. K. (2019). Metabolic targets in nonalcoholic fatty liver disease. *Cell Mol Gastroenterol Hepatol*, 8(2), 247-267. doi:10.1016/j.jcmgh.2019.04.007. **PMC6698700**.
 113. Sunami, Y. (2020). NASH, fibrosis and hepatocellular carcinoma: Lipid synthesis and glutamine/acetate signaling. *Int J Mol Sci*, 21(18). doi:10.3390/ijms21186799. **PMID: 32947972**.
 114. Stiede, K., Miao, W., Blanchette, H. S., Beysen, C., Harriman, G., Harwood, H. J., Jr., Kelley, H., Kapeller, R., Schmalbach, T., & Westlin, W. F. (2017). Acetyl-coenzyme A carboxylase inhibition reduces de novo lipogenesis in overweight male subjects: A randomized, double-blind, crossover study. *Hepatology*, 66(2), 324-334. doi:10.1002/hep.29246. **PMC5599970**.
 115. El, M., & Zilversmit, D. B. (1958). Fate of triglycerides and phospholipids of lymph and artificial fat emulsions: disappearance from the circulation.

- Am J Physiol*, 193(2), 294-300. doi:10.1152/ajplegacy.1958.193.2.294.
PMID: 13533545.
116. Feldstein, A. E., Charatcharoenwitthaya, P., Treeprasertsuk, S., Benson, J. T., Enders, F. B., & Angulo, P. (2009). The natural history of non-alcoholic fatty liver disease in children: a follow-up study for up to 20 years. *Gut*, 58(11), 1538-1544. doi:10.1136/gut.2008.171280.
PMC2792743.
 117. Harlow, K. E., Africa, J. A., Wells, A., Belt, P. H., Behling, C. A., Jain, A. K., Molleston, J. P., Newton, K. P., Rosenthal, P., Vos, M. B., Xanthakos, S. A., Lavine, J. E., Schwimmer, J. B., & Nonalcoholic Steatohepatitis Clinical Research, N. (2018). Clinically actionable hypercholesterolemia and hypertriglyceridemia in children with nonalcoholic fatty liver disease. *J Pediatr*, 198, 76-83 e72. doi:10.1016/j.jpeds.2018.02.038. **PMC6019181.**
 118. Alves-Bezerra, M., & Cohen, D. E. (2017). Triglyceride metabolism in the liver. *Compr Physiol*, 8(1), 1-8. doi:10.1002/cphy.c170012.
PMC6376873.
 119. Horton, J. D., Goldstein, J. L., & Brown, M. S. (2002). SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest*, 109(9), 1125-1131. doi:10.1172/JCI15593. **PMC150968.**
 120. Luo, J., Yang, H., & Song, B. L. (2020). Mechanisms and regulation of cholesterol homeostasis. *Nat Rev Mol Cell Biol*, 21(4), 225-245. doi:10.1038/s41580-019-0190-7. **PMID: 31848472.**
 121. Caballero, F., Fernandez, A., De Lacy, A. M., Fernandez-Checa, J. C., Caballeria, J., & Garcia-Ruiz, C. (2009). Enhanced free cholesterol, SREBP-2 and StAR expression in human NASH. *J Hepatol*, 50(4), 789-796. doi:10.1016/j.jhep.2008.12.016. **PMID: 19231010.**
 122. Van Rooyen, D. M., Larter, C. Z., Haigh, W. G., Yeh, M. M., Ioannou, G., Kuver, R., Lee, S. P., Teoh, N. C., & Farrell, G. C. (2011). Hepatic free cholesterol accumulates in obese, diabetic mice and causes nonalcoholic steatohepatitis. *Gastroenterology*, 141(4), 1393-1403, 1403 e1391-1395. doi:10.1053/j.gastro.2011.06.040. **PMC3186822.**
 123. Arteel, G. E. (2012). Beyond reasonable doubt: who is the culprit in lipotoxicity in NAFLD/NASH? *Hepatology*, 55(6), 2030-2032. doi:10.1002/hep.25721. **PMC5047035.**
 124. Kerr, T. A., & Davidson, N. O. (2012). Cholesterol and nonalcoholic fatty liver disease: renewed focus on an old villain. *Hepatology*, 56(5), 1995-1998. doi:10.1002/hep.26088. **PMC3627394.**
 125. Arguello, G., Balboa, E., Arrese, M., & Zanlungo, S. (2015). Recent insights on the role of cholesterol in non-alcoholic fatty liver disease. *Biochim Biophys Acta*, 1852(9), 1765-1778. doi:10.1016/j.bbadis.2015.05.015. **PMID: 26027904.**
 126. Ioannou, G. N. (2016). The role of cholesterol in the pathogenesis of NASH. *Trends Endocrinol Metab*, 27(2), 84-95. doi:10.1016/j.tem.2015.11.008. **PMID: 26703097.**

127. Farrell, G. (2014). Should we lower lipids in nonalcoholic fatty liver disease? *Clin Gastroenterol Hepatol*, 12(1), 152-155. doi:10.1016/j.cgh.2013.07.041. **PMID: 23958558**.
128. Van Rooyen, D. M., Gan, L. T., Yeh, M. M., Haigh, W. G., Larter, C. Z., Ioannou, G., Teoh, N. C., & Farrell, G. C. (2013). Pharmacological cholesterol lowering reverses fibrotic NASH in obese, diabetic mice with metabolic syndrome. *J Hepatol*, 59(1), 144-152. doi:10.1016/j.jhep.2013.02.024. **PMID: 23500152**
129. Wurie, H. R., Buckett, L., & Zammit, V. A. (2012). Diacylglycerol acyltransferase 2 acts upstream of diacylglycerol acyltransferase 1 and utilizes nascent diglycerides and de novo synthesized fatty acids in HepG2 cells. *FEBS J*, 279(17), 3033-3047. doi:10.1111/j.1742-4658.2012.08684.x. **PMID: 22748069**.
130. Yen, C. L., Stone, S. J., Koliwad, S., Harris, C., & Farese, R. V., Jr. (2008). Thematic review series: glycerolipids. DGAT enzymes and triacylglycerol biosynthesis. *J Lipid Res*, 49(11), 2283-2301. doi:10.1194/jlr.R800018-JLR200. **PMC3837458**.
131. Lewis, G. F., Uffelman, K. D., Szeto, L. W., Weller, B., & Steiner, G. (1995). Interaction between free fatty acids and insulin in the acute control of very low density lipoprotein production in humans. *J Clin Invest*, 95(1), 158-166. doi:10.1172/JCI117633. **PMC295395**.
132. Lewis, G. F., & Steiner, G. (1996). Acute effects of insulin in the control of VLDL production in humans. Implications for the insulin-resistant state. *Diabetes Care*, 19(4), 390-393. doi:10.2337/diacare.19.4.390. **PMID: 8729170**.
133. Taghibiglou, C., Carpentier, A., Van Iderstine, S. C., Chen, B., Rudy, D., Aiton, A., Lewis, G. F., & Adeli, K. (2000). Mechanisms of hepatic very low density lipoprotein overproduction in insulin resistance. Evidence for enhanced lipoprotein assembly, reduced intracellular ApoB degradation, and increased microsomal triglyceride transfer protein in a fructose-fed hamster model. *J Biol Chem*, 275(12), 8416-8425. doi:10.1074/jbc.275.12.8416. **PMID: 10722675**.
134. Adeli, K., Taghibiglou, C., Van Iderstine, S. C., & Lewis, G. F. (2001). Mechanisms of hepatic very low-density lipoprotein overproduction in insulin resistance. *Trends Cardiovasc Med*, 11(5), 170-176. doi:10.1016/s1050-1738(01)00084-6. **PMID: 11597827**.
135. Poulsen, M. K., Nellemann, B., Stodkilde-Jorgensen, H., Pedersen, S. B., Gronbaek, H., & Nielsen, S. (2016). Impaired insulin suppression of VLDL-triglyceride kinetics in nonalcoholic fatty liver disease. *J Clin Endocrinol Metab*, 101(4), 1637-1646. doi:10.1210/jc.2015-3476. **PMID: 26829441**
136. Lytle, K. A., Bush, N. C., Triay, J. M., Kellogg, T. A., Kendrick, M. L., Swain, J. M., Gathaiya, N. W., Hames, K. C., & Jensen, M. D. (2019). Hepatic fatty acid balance and hepatic fat content in humans with severe obesity. *J Clin Endocrinol Metab*, 104(12), 6171-6181. doi:10.1210/jc.2019-00875. **PMC6821207**.

137. Mittendorfer, B., Yoshino, M., Patterson, B. W., & Klein, S. (2016). VLDL triglyceride kinetics in lean, overweight, and obese men and women. *J Clin Endocrinol Metab*, *101*(11), 4151-4160. doi:10.1210/jc.2016-1500. **PMC5095238**.
138. Fujita, K., Nozaki, Y., Wada, K., Yoneda, M., Fujimoto, Y., Fujitake, M., Endo, H., Takahashi, H., Inamori, M., Kobayashi, N., Kirikoshi, H., Kubota, K., Saito, S., & Nakajima, A. (2009). Dysfunctional very-low-density lipoprotein synthesis and release is a key factor in nonalcoholic steatohepatitis pathogenesis. *Hepatology*, *50*(3), 772-780. doi:10.1002/hep.23094. **PMID: 19650159**.
139. Hussain, M. M., Rava, P., Walsh, M., Rana, M., & Iqbal, J. (2012). Multiple functions of microsomal triglyceride transfer protein. *Nutr Metab (Lond)*, *9*, 14. doi:10.1186/1743-7075-9-14. **PMC3337244**.
140. Charlton, M., Sreekumar, R., Rasmussen, D., Lindor, K., & Nair, K. S. (2002). Apolipoprotein synthesis in nonalcoholic steatohepatitis. *Hepatology*, *35*(4), 898-904. doi:10.1053/jhep.2002.32527. **PMID: 11915037**.
141. Smith, G. I., Magkos, F., Reeds, D. N., Okunade, A. L., Patterson, B. W., & Mittendorfer, B. (2013). One day of mixed meal overfeeding reduces hepatic insulin sensitivity and increases VLDL particle but not VLDL-triglyceride secretion in overweight and obese men. *J Clin Endocrinol Metab*, *98*(8), 3454-3462. doi:10.1210/jc.2013-1786. **PMC3733854**.
142. Wakil, S. J., & Abu-Elheiga, L. A. (2009). Fatty acid metabolism: target for metabolic syndrome. *J Lipid Res*, *50 Suppl*, S138-143. doi:10.1194/jlr.R800079-JLR200. **PMC2674721**.
143. Fritz, I. (1955). The effect of muscle extracts on the oxidation of palmitic acid by liver slices and homogenates. *Acta Physiol Scand*, *34*(4), 367-385. doi:10.1111/j.1748-1716.1955.tb01256.x. **PMID: 13282744**.
144. Fritz, I. B., & Yue, K. T. (1963). Long-chain carnitine acyltransferase and the role of acylcarnitine derivatives in the catalytic increase of fatty acid oxidation induced by carnitine. *J Lipid Res*, *4*, 279-288. **PMID: 14168165**.
145. Bremer, J. (1963). Carnitine in intermediary metabolism. The biosynthesis of palmitylcarnitine by cell subfractions. *J Biol Chem*, *238*, 2774-2779. **PMID: 14063302**.
146. McGarry, J. D., & Brown, N. F. (1997). The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. *Eur J Biochem*, *244*(1), 1-14. doi:10.1111/j.1432-1033.1997.00001.x. **PMID: 9063439**.
147. Bartlett, K., & Eaton, S. (2004). Mitochondrial beta-oxidation. *Eur J Biochem*, *271*(3), 462-469. doi:10.1046/j.1432-1033.2003.03947.x. **PMID: 14728673**.
148. Eaton, S., Bartlett, K., & Pourfarzam, M. (1996). Mammalian mitochondrial beta-oxidation. *Biochem J*, *320 (Pt 2)*, 345-357. doi:10.1042/bj3200345. **PMC1217938**.
149. Rector, R. S., Payne, R. M., & Ibdah, J. A. (2008). Mitochondrial trifunctional protein defects: clinical implications and therapeutic

- approaches. *Adv Drug Deliv Rev*, 60(13-14), 1488-1496.
doi:10.1016/j.addr.2008.04.014. **PMC2848452**.
150. Angdisen, J., Moore, V. D., Cline, J. M., Payne, R. M., & Ibdah, J. A. (2005). Mitochondrial trifunctional protein defects: molecular basis and novel therapeutic approaches. *Curr Drug Targets Immune Endocr Metabol Disord*, 5(1), 27-40. doi:10.2174/1568008053174796. **PMID: 15777202**.
 151. Eaton, S., Bursby, T., Middleton, B., Pourfarzam, M., Mills, K., Johnson, A. W., & Bartlett, K. (2000). The mitochondrial trifunctional protein: centre of a beta-oxidation metabolon? *Biochem Soc Trans*, 28(2), 177-182. doi:10.1042/bst0280177. **PMID: 10816122**.
 152. Dasarathy, S., Yang, Y., McCullough, A. J., Marczewski, S., Bennett, C., & Kalhan, S. C. (2011). Elevated hepatic fatty acid oxidation, high plasma fibroblast growth factor 21, and fasting bile acids in nonalcoholic steatohepatitis. *Eur J Gastroenterol Hepatol*, 23(5), 382-388. doi:10.1097/MEG.0b013e328345c8c7. **PMC3493151**.
 153. Dasarathy, S., Kasumov, T., Edmison, J. M., Gruca, L. L., Bennett, C., Duenas, C., Marczewski, S., McCullough, A. J., Hanson, R. W., & Kalhan, S. C. (2009). Glycine and urea kinetics in nonalcoholic steatohepatitis in human: effect of intralipid infusion. *Am J Physiol Gastrointest Liver Physiol*, 297(3), G567-575. doi:10.1152/ajpgi.00042.2009. **PMC2739817**.
 154. Sanyal, A. J., Campbell-Sargent, C., Mirshahi, F., Rizzo, W. B., Contos, M. J., Sterling, R. K., Luketic, V. A., Shiffman, M. L., & Clore, J. N. (2001). Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. *Gastroenterology*, 120(5), 1183-1192. doi:10.1053/gast.2001.23256. **PMID: 11266382**
 155. Miele, L., Grieco, A., Armuzzi, A., Candelli, M., Forgione, A., Gasbarrini, A., & Gasbarrini, G. (2003). Hepatic mitochondrial beta-oxidation in patients with nonalcoholic steatohepatitis assessed by ¹³C-octanoate breath test. *Am J Gastroenterol*, 98(10), 2335-2336. doi:10.1111/j.1572-0241.2003.07725.x. **PMID: 14572600**
 156. Reddy, J. K., & Rao, M. S. (2006). Lipid metabolism and liver inflammation. II. Fatty liver disease and fatty acid oxidation. *Am J Physiol Gastrointest Liver Physiol*, 290(5), G852-858. doi:10.1152/ajpgi.00521.2005. **PMID: 16603729**.
 157. Croci, I., Byrne, N. M., Choquette, S., Hills, A. P., Chachay, V. S., Clouston, A. D., O'Moore-Sullivan, T. M., Macdonald, G. A., Prins, J. B., & Hickman, I. J. (2013). Whole-body substrate metabolism is associated with disease severity in patients with non-alcoholic fatty liver disease. *Gut*, 62(11), 1625-1633. doi:10.1136/gutjnl-2012-302789. **PMID: 23077135**.
 158. Fletcher, J. A., Deja, S., Satapati, S., Fu, X., Burgess, S. C., & Browning, J. D. (2019). Impaired ketogenesis and increased acetyl-CoA oxidation promote hyperglycemia in human fatty liver. *JCI Insight*, 5. doi:10.1172/jci.insight.127737. **PMC6629163**.

159. Lee, J., Choi, J., Selen Alpergin, E. S., Zhao, L., Hartung, T., Scafidi, S., Riddle, R. C., & Wolfgang, M. J. (2017). Loss of hepatic mitochondrial long-chain fatty acid oxidation confers resistance to diet-induced obesity and glucose intolerance. *Cell Rep*, *20*(3), 655-667. doi:10.1016/j.celrep.2017.06.080. **PMC5546239**.
160. Softic, S., Meyer, J. G., Wang, G. X., Gupta, M. K., Batista, T. M., Lauritzen, H., Fujisaka, S., Serra, D., Herrero, L., Willoughby, J., Fitzgerald, K., Ilkayeva, O., Newgard, C. B., Gibson, B. W., Schilling, B., Cohen, D. E., & Kahn, C. R. (2019). Dietary sugars alter hepatic fatty acid oxidation via transcriptional and post-translational modifications of mitochondrial proteins. *Cell Metab*, *30*(4), 735-753 e734. doi:10.1016/j.cmet.2019.09.003. **PMID: 31577934**.
161. Kotronen, A., Seppala-Lindroos, A., Vehkavaara, S., Bergholm, R., Frayn, K. N., Fielding, B. A., & Yki-Jarvinen, H. (2009). Liver fat and lipid oxidation in humans. *Liver Int*, *29*(9), 1439-1446. doi:10.1111/j.1478-3231.2009.02076.x. **PMID: 19602132**.
162. Gastaldelli, A., Cusi, K., Pettiti, M., Hardies, J., Miyazaki, Y., Berria, R., Buzzigoli, E., Sironi, A. M., Cersosimo, E., Ferrannini, E., & DeFronzo, R. A. (2007). Relationship between hepatic/visceral fat and hepatic insulin resistance in nondiabetic and type 2 diabetic subjects. *Gastroenterology*, *133*(2), 496-506. doi:10.1053/j.gastro.2007.04.068. **PMID: 17681171**.
163. Naguib, G., Morris, N., Yang, S., Fryzek, N., Haynes-Williams, V., Huang, W. A., Norman-Wheeler, J., & Rotman, Y. (2020). Dietary fatty acid oxidation is decreased in non-alcoholic fatty liver disease: A palmitate breath test study. *Liver Int*, *40*(3), 590-597. doi:10.1111/liv.14309. **PMC7056545**.
164. Nassir, F., Arndt, J. J., Johnson, S. A., & Ibdah, J. A. (2018). Regulation of mitochondrial trifunctional protein modulates nonalcoholic fatty liver disease in mice. *J Lipid Res*, *59*(6), 967-973. doi:10.1194/jlr.M080952. **PMC5983392**.
165. McGarry, J. D., Mannaerts, G. P., & Foster, D. W. (1977). A possible role for malonyl-CoA in the regulation of hepatic fatty acid oxidation and ketogenesis. *J Clin Invest*, *60*(1), 265-270. doi:10.1172/JC1108764. **PMC372365**.
166. Abu-Elheiga, L., Matzuk, M. M., Abo-Hashema, K. A., & Wakil, S. J. (2001). Continuous fatty acid oxidation and reduced fat storage in mice lacking acetyl-CoA carboxylase 2. *Science*, *291*(5513), 2613-2616. doi:10.1126/science.1056843. **PMID: 11283375**.
167. Qu, Q., Zeng, F., Liu, X., Wang, Q. J., & Deng, F. (2016). Fatty acid oxidation and carnitine palmitoyltransferase I: emerging therapeutic targets in cancer. *Cell Death Dis*, *7*, e2226. doi:10.1038/cddis.2016.132. **PMC4917665**.
168. Zhu, L., Baker, S. S., Liu, W., Tao, M. H., Patel, R., Nowak, N. J., & Baker, R. D. (2011). Lipid in the livers of adolescents with nonalcoholic steatohepatitis: combined effects of pathways on steatosis. *Metabolism*, *60*(7), 1001-1011. doi:10.1016/j.metabol.2010.10.003. **PMID: 21075404**

169. Mitsuyoshi, H., Yasui, K., Harano, Y., Endo, M., Tsuji, K., Minami, M., Itoh, Y., Okanoue, T., & Yoshikawa, T. (2009). Analysis of hepatic genes involved in the metabolism of fatty acids and iron in nonalcoholic fatty liver disease. *Hepatol Res*, 39(4), 366-373. doi:10.1111/j.1872-034X.2008.00464.x. **PMID: 19054139**
170. Dorn, C., Riener, M. O., Kirovski, G., Saugspier, M., Steib, K., Weiss, T. S., Gabele, E., Kristiansen, G., Hartmann, A., & Hellerbrand, C. (2010). Expression of fatty acid synthase in nonalcoholic fatty liver disease. *Int J Clin Exp Pathol*, 3(5), 505-514. **PMC2897101**.
171. Nagaya, T., Tanaka, N., Kimura, T., Kitabatake, H., Fujimori, N., Komatsu, M., Horiuchi, A., Yamaura, T., Umemura, T., Sano, K., Gonzalez, F. J., Aoyama, T., & Tanaka, E. (2015). Mechanism of the development of nonalcoholic steatohepatitis after pancreaticoduodenectomy. *BBA Clin*, 3, 168-174. doi:10.1016/j.bbacli.2015.02.001. **PMC4661550**.
172. Kohjima, M., Enjoji, M., Higuchi, N., Kato, M., Kotoh, K., Yoshimoto, T., Fujino, T., Yada, M., Yada, R., Harada, N., Takayanagi, R., & Nakamuta, M. (2007). Re-evaluation of fatty acid metabolism-related gene expression in nonalcoholic fatty liver disease. *Int J Mol Med*, 20(3), 351-358. **PMID: 17671740**.
173. Greco, D., Kotronen, A., Westerbacka, J., Puig, O., Arkkila, P., Kiviluoto, T., Laitinen, S., Kolak, M., Fisher, R. M., Hamsten, A., Auvinen, P., & Yki-Jarvinen, H. (2008). Gene expression in human NAFLD. *Am J Physiol Gastrointest Liver Physiol*, 294(5), G1281-1287. doi:10.1152/ajpgi.00074.2008. **PMID: 18388185**.
174. Rong, S., Cortes, V. A., Rashid, S., Anderson, N. N., McDonald, J. G., Liang, G., Moon, Y. A., Hammer, R. E., & Horton, J. D. (2017). Expression of SREBP-1c requires SREBP-2-mediated generation of a sterol ligand for LXR in livers of mice. *Elife*, 6. doi:10.7554/eLife.25015. **PMC5348127**.
175. Ahmed, M. H., & Byrne, C. D. (2007). Modulation of sterol regulatory element binding proteins (SREBPs) as potential treatments for non-alcoholic fatty liver disease (NAFLD). *Drug Discov Today*, 12(17-18), 740-747. doi:10.1016/j.drudis.2007.07.009. **PMID: 17826687**.
176. Westerbacka, J., Kolak, M., Kiviluoto, T., Arkkila, P., Siren, J., Hamsten, A., Fisher, R. M., & Yki-Jarvinen, H. (2007). Genes involved in fatty acid partitioning and binding, lipolysis, monocyte/macrophage recruitment, and inflammation are overexpressed in the human fatty liver of insulin-resistant subjects. *Diabetes*, 56(11), 2759-2765. doi:10.2337/db07-0156. **PMID: 17704301**
177. Bechmann, L. P., Gieseler, R. K., Sowa, J. P., Kahraman, A., Erhard, J., Wedemeyer, I., Emons, B., Jochum, C., Feldkamp, T., Gerken, G., & Canbay, A. (2010). Apoptosis is associated with CD36/fatty acid translocase upregulation in non-alcoholic steatohepatitis. *Liver Int*, 30(6), 850-859. doi:10.1111/j.1478-3231.2010.02248.x. **PMID: 20408954**.
178. Nakamuta, M., Fujino, T., Yada, R., Yada, M., Yasutake, K., Yoshimoto, T., Harada, N., Higuchi, N., Kato, M., Kohjima, M., Taketomi, A., Maehara, Y.,

- Nakashima, M., Kotoh, K., & Enjoji, M. (2009). Impact of cholesterol metabolism and the LXRA α -SREBP-1c pathway on nonalcoholic fatty liver disease. *Int J Mol Med*, 23(5), 603-608. doi:10.3892/ijmm_00000170. **PMID: 19360318**
179. Koliaki, C., Szendroedi, J., Kaul, K., Jelenik, T., Nowotny, P., Jankowiak, F., Herder, C., Carstensen, M., Krausch, M., Knoefel, W. T., Schlensak, M., & Roden, M. (2015). Adaptation of hepatic mitochondrial function in humans with non-alcoholic fatty liver is lost in steatohepatitis. *Cell Metab*, 21(5), 739-746. doi:10.1016/j.cmet.2015.04.004. **PMID: 25955209**
 180. Desterke, C., & Chiappini, F. (2019). Lipid related genes altered in NASH connect inflammation in liver pathogenesis progression to HCC: A canonical pathway. *Int J Mol Sci*, 20(22). doi:10.3390/ijms20225594. **PMC6888337**.
 181. Miquilena-Colina, M. E., Lima-Cabello, E., Sanchez-Campos, S., Garcia-Mediavilla, M. V., Fernandez-Bermejo, M., Lozano-Rodriguez, T., Vargas-Castrillon, J., Buque, X., Ochoa, B., Aspichueta, P., Gonzalez-Gallego, J., & Garcia-Monzon, C. (2011). Hepatic fatty acid translocase CD36 upregulation is associated with insulin resistance, hyperinsulinaemia and increased steatosis in non-alcoholic steatohepatitis and chronic hepatitis C. *Gut*, 60(10), 1394-1402. doi:10.1136/gut.2010.222844. **PMID: 21270117**.
 182. Parks, E. J., Skokan, L. E., Timlin, M. T., & Dingfelder, C. S. (2008). Dietary sugars stimulate fatty acid synthesis in adults. *J Nutr*, 138(6), 1039-1046. doi:10.1093/jn/138.6.1039. **PMC2546703**.
 183. 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. doi:10.1093/ajcn/81.1.35. **PMID: 15640457**.
 184. Schwarz, J. M., Clearfield, M., & Mulligan, K. (2017). Conversion of sugar to fat: Is hepatic de novo lipogenesis leading to metabolic syndrome and associated chronic diseases? *J Am Osteopath Assoc*, 117(8), 520-527. doi:10.7556/jaoa.2017.102. **PMID: 28759094**.
 185. Lambert, J. E., Ryan, E. A., Thomson, A. B., & Clandinin, M. T. (2013). De novo lipogenesis and cholesterol synthesis in humans with long-standing type 1 diabetes are comparable to non-diabetic individuals. *PLoS One*, 8(12), e82530. doi:10.1371/journal.pone.0082530. **PMC3871159**.
 186. Rosqvist, F., McNeil, C. A., Pramfalk, C., Parry, S. A., Low, W. S., Cornfield, T., Fielding, B. A., & Hodson, L. (2019). Fasting hepatic de novo lipogenesis is not reliably assessed using circulating fatty acid markers. *Am J Clin Nutr*, 109(2), 260-268. doi:10.1093/ajcn/nqy304. **PMC6367991**.
 187. Roumans, K. H. M., Lindeboom, L., Veeraiyah, P., Remie, C. M. E., Phielix, E., Havekes, B., Bruls, Y. M. H., Brouwers, M., Stahlman, M., Alsema, M., Peters, H. P. F., de Mutsert, R., Staels, B., Taskinen, M. R., Boren, J., Schrauwen, P., & Schrauwen-Hinderling, V. B. (2020). Hepatic saturated fatty acid fraction is associated with de novo lipogenesis and hepatic

- insulin resistance. *Nat Commun*, 11(1), 1891. doi:10.1038/s41467-020-15684-0. **PMC7170906**.
188. Smith, G. I., Shankaran, M., Yoshino, M., Schweitzer, G. G., Chondronikola, M., Beals, J. W., Okunade, A. L., Patterson, B. W., Nyangau, E., Field, T., Sirlin, C. B., Talukdar, S., Hellerstein, M. K., & Klein, S. (2020). Insulin resistance drives hepatic de novo lipogenesis in nonalcoholic fatty liver disease. *J Clin Invest*, 130(3), 1453-1460. doi:10.1172/JCI134165. **PMC7269561**.
189. Syed-Abdul, M. M., Le, N. T., Jacome-Sosa, M., Hu, Q., Oxler, B. M., Bingham, K., Arreola, R., Juboori, A. M. A., Gaballah, A. H., Bartholow, B. D., Ibdah, J. A., & Parks, E. J. (2018). Tailgate study: A pilot study measuring the impact of food and alcohol intake on whole-body and liver metabolism. *FASEB Journal*, 32(S760.6).
190. Das, S. K., Mondal, A. K., & Elbein, S. C. (2010). Distinct gene expression profiles characterize cellular responses to palmitate and oleate. *J Lipid Res*, 51(8), 2121-2131. doi:10.1194/jlr.M004275. **PMC2903813**.
191. Pierce, A. A., Pickens, M. K., Siao, K., Grenert, J. P., & Maher, J. J. (2015). Differential hepatotoxicity of dietary and DNL-derived palmitate in the methionine-choline-deficient model of steatohepatitis. *BMC Gastroenterol*, 15, 72. doi:10.1186/s12876-015-0298-y. **PMC4479079**.
192. Girard, J., Ferre, P., & Foufelle, F. (1997). Mechanisms by which carbohydrates regulate expression of genes for glycolytic and lipogenic enzymes. *Annu Rev Nutr*, 17, 325-352. doi:10.1146/annurev.nutr.17.1.325. **PMID: 9240931**
193. Towle, H. C. (1995). Metabolic regulation of gene transcription in mammals. *J Biol Chem*, 270(40), 23235-23238. doi:10.1074/jbc.270.40.23235. **PMID: 7559472**.
194. Bril, F., Lomonaco, R., Orsak, B., Ortiz-Lopez, C., Webb, A., Tio, F., Hecht, J., & Cusi, K. (2014). Relationship between disease severity, hyperinsulinemia, and impaired insulin clearance in patients with nonalcoholic steatohepatitis. *Hepatology*, 59(6), 2178-2187. doi:10.1002/hep.26988. **PMID: 24777953**
195. Kleiner, D. E., Brunt, E. M., Van Natta, M., Behling, C., Contos, M. J., Cummings, O. W., Ferrell, L. D., Liu, Y. C., Torbenson, M. S., Unalp-Arida, A., Yeh, M., McCullough, A. J., Sanyal, A. J., & Nonalcoholic Steatohepatitis Clinical Research, N. (2005). Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology*, 41(6), 1313-1321. doi:10.1002/hep.20701. **PMID: 15915461**.
196. Brunt, E. M., Kleiner, D. E., Wilson, L. A., Belt, P., Neuschwander-Tetri, B. A., & Network, N. C. R. (2011). Nonalcoholic fatty liver disease (NAFLD) activity score and the histopathologic diagnosis in NAFLD: distinct clinicopathologic meanings. *Hepatology*, 53(3), 810-820. doi:10.1002/hep.24127. **PMC3079483**.

197. Hellerstein, M. K., & Neese, R. A. (1992). Mass isotopomer distribution analysis: a technique for measuring biosynthesis and turnover of polymers. *Am J Physiol*, 263(5 Pt 1), E988-1001. doi:10.1152/ajpendo.1992.263.5.E988. **PMID: 1443132.**
198. Hellerstein, M. K., Christiansen, M., Kaempfer, S., Kletke, C., Wu, K., Reid, J. S., Mulligan, K., Hellerstein, N. S., & Shackleton, C. H. (1991). Measurement of de novo hepatic lipogenesis in humans using stable isotopes. *J Clin Invest*, 87(5), 1841-1852. doi:10.1172/JCI115206. **PMC295308.**
199. Hellerstein, M. K., Kletke, C., Kaempfer, S., Wu, K., & Shackleton, C. H. (1991). Use of mass isotopomer distributions in secreted lipids to sample lipogenic acetyl-CoA pool in vivo in humans. *Am J Physiol*, 261(4 Pt 1), E479-486. doi:10.1152/ajpendo.1991.261.4.E479. **PMID: 1928339.**
200. Antoniewicz, M. R. (2018). A guide to (13)C metabolic flux analysis for the cancer biologist. *Exp Mol Med*, 50(4), 19. doi:10.1038/s12276-018-0060-y. **PMC5938039.**
201. Chong, M. F., Fielding, B. A., & Frayn, K. N. (2007). Mechanisms for the acute effect of fructose on postprandial lipemia. *Am J Clin Nutr*, 85(6), 1511-1520. doi:10.1093/ajcn/85.6.1511. **PMID: 17556686.**
202. Hellerstein, M. K. (1991). Relationship between precursor enrichment and ratio of excess M2/excess M1 isotopomer frequencies in a secreted polymer. *J Biol Chem*, 266(17), 10920-10924. **PMID: 2040609.**
203. Hudgins, L. C., Hellerstein, M., Seidman, C., Neese, R., Diakun, J., & Hirsch, J. (1996). Human fatty acid synthesis is stimulated by a eucaloric low fat, high carbohydrate diet. *J Clin Invest*, 97(9), 2081-2091. doi:10.1172/JCI118645. **PMC507283.**
204. Chong, M. F., Hodson, L., Bickerton, A. S., Roberts, R., Neville, M., Karpe, F., Frayn, K. N., & Fielding, B. A. (2008). Parallel activation of de novo lipogenesis and stearoyl-CoA desaturase activity after 3 d of high-carbohydrate feeding. *Am J Clin Nutr*, 87(4), 817-823. doi:10.1093/ajcn/87.4.817. **PMID: 18400702.**
205. Knebel, B., Fahlbusch, P., Dille, M., Wahlers, N., Hartwig, S., Jacob, S., Kettel, U., Schiller, M., Herebian, D., Koellmer, C., Lehr, S., Muller-Wieland, D., & Kotzka, J. (2019). Fatty liver due to increased de novo lipogenesis: Alterations in the hepatic peroxisomal proteome. *Front Cell Dev Biol*, 7, 248. doi:10.3389/fcell.2019.00248. **PMC6823594.**
206. Sjogren, P., Sierra-Johnson, J., Gertow, K., Rosell, M., Vessby, B., de Faire, U., Hamsten, A., Hellenius, M. L., & Fisher, R. M. (2008). Fatty acid desaturases in human adipose tissue: relationships between gene expression, desaturation indexes and insulin resistance. *Diabetologia*, 51(2), 328-335. doi:10.1007/s00125-007-0876-9. **PMID: 18030445.**
207. Warensjo, E., Ohrvall, M., & Vessby, B. (2006). Fatty acid composition and estimated desaturase activities are associated with obesity and lifestyle variables in men and women. *Nutr Metab Cardiovasc Dis*, 16(2), 128-136. doi:10.1016/j.numecd.2005.06.001. **PMID: 16487913.**

208. Flowers, M. T. (2009). The delta9 fatty acid desaturation index as a predictor of metabolic disease. *Clin Chem*, 55(12), 2071-2073. doi:10.1373/clinchem.2009.135152. **PMID: 19850628.**
209. Yee, J. K., Lee, W. N., Han, G., Ross, M. G., & Desai, M. (2011). Organ-specific alterations in fatty acid de novo synthesis and desaturation in a rat model of programmed obesity. *Lipids Health Dis*, 10, 72. doi:10.1186/1476-511X-10-72. **PMC3112422.**
210. Cedernaes, J., Alsio, J., Vastermark, A., Riserus, U., & Schioth, H. B. (2013). Adipose tissue stearoyl-CoA desaturase 1 index is increased and linoleic acid is decreased in obesity-prone rats fed a high-fat diet. *Lipids Health Dis*, 12, 2. doi:10.1186/1476-511X-12-2. **PMC3558438.**
211. Green, C. D., Ozguden-Akkoc, C. G., Wang, Y., Jump, D. B., & Olson, L. K. (2010). Role of fatty acid elongases in determination of de novo synthesized monounsaturated fatty acid species. *J Lipid Res*, 51(7), 1871-1877. doi:10.1194/jlr.M004747. **PMC2882732.**
212. Silbernagel, G., Kovarova, M., Cegan, A., Machann, J., Schick, F., Lehmann, R., Haring, H. U., Stefan, N., Schleicher, E., Fritsche, A., & Peter, A. (2012). High hepatic SCD1 activity is associated with low liver fat content in healthy subjects under a lipogenic diet. *J Clin Endocrinol Metab*, 97(12), E2288-2292. doi:10.1210/jc.2012-2152. **PMID: 23015656.**
213. Peter, A., Cegan, A., Wagner, S., Elcnerova, M., Konigsrainer, A., Konigsrainer, I., Haring, H. U., Schleicher, E. D., & Stefan, N. (2011). Relationships between hepatic stearoyl-CoA desaturase-1 activity and mRNA expression with liver fat content in humans. *Am J Physiol Endocrinol Metab*, 300(2), E321-326. doi:10.1152/ajpendo.00306.2010. **PMID: 21045174.**

TABLE 1.1 REFERENCES

1. Zhu, L., Baker, S. S., Liu, W., Tao, M. H., Patel, R., Nowak, N. J., & Baker, R. D. (2011). Lipid in the livers of adolescents with nonalcoholic steatohepatitis: combined effects of pathways on steatosis. *Metabolism*, *60*(7), 1001-1011. doi:10.1016/j.metabol.2010.10.003. **PMID: 21075404**
2. Mitsuyoshi, H., Yasui, K., Harano, Y., Endo, M., Tsuji, K., Minami, M., Itoh, Y., Okanoue, T., & Yoshikawa, T. (2009). Analysis of hepatic genes involved in the metabolism of fatty acids and iron in nonalcoholic fatty liver disease. *Hepatol Res*, *39*(4), 366-373. doi:10.1111/j.1872-034X.2008.00464.x. **PMID: 19054139**
3. Westerbacka, J., Kolak, M., Kiviluoto, T., Arkkila, P., Siren, J., Hamsten, A., Fisher, R. M., & Yki-Jarvinen, H. (2007). Genes involved in fatty acid partitioning and binding, lipolysis, monocyte/macrophage recruitment, and inflammation are overexpressed in the human fatty liver of insulin-resistant subjects. *Diabetes*, *56*(11), 2759-2765. doi:10.2337/db07-0156. **PMID: 17704301**
4. Bechmann, L. P., Gieseler, R. K., Sowa, J. P., Kahraman, A., Erhard, J., Wedemeyer, I., Emons, B., Jochum, C., Feldkamp, T., Gerken, G., & Canbay, A. (2010). Apoptosis is associated with CD36/fatty acid translocase upregulation in non-alcoholic steatohepatitis. *Liver Int*, *30*(6), 850-859. doi:10.1111/j.1478-3231.2010.02248.x. **PMID: 20408954**.
5. Miquilena-Colina, M. E., Lima-Cabello, E., Sanchez-Campos, S., Garcia-Mediavilla, M. V., Fernandez-Bermejo, M., Lozano-Rodriguez, T., Vargas-Castrillon, J., Buque, X., Ochoa, B., Aspichueta, P., Gonzalez-Gallego, J., & Garcia-Monzon, C. (2011). Hepatic fatty acid translocase CD36 upregulation is associated with insulin resistance, hyperinsulinaemia and increased steatosis in non-alcoholic steatohepatitis and chronic hepatitis C. *Gut*, *60*(10), 1394-1402. doi:10.1136/gut.2010.222844. **PMID: 21270117**.
6. Desterke, C., & Chiappini, F. (2019). Lipid related genes altered in NASH connect inflammation in liver pathogenesis progression to HCC: A canonical pathway. *Int J Mol Sci*, *20*(22). doi:10.3390/ijms20225594. **PMC6888337**.
7. Greco, D., Kotronen, A., Westerbacka, J., Puig, O., Arkkila, P., Kiviluoto, T., Laitinen, S., Kolak, M., Fisher, R. M., Hamsten, A., Auvinen, P., & Yki-Jarvinen, H. (2008). Gene expression in human NAFLD. *Am J Physiol Gastrointest Liver Physiol*, *294*(5), G1281-1287. doi:10.1152/ajpgi.00074.2008. **PMID: 18388185**.
8. Auguet, T., Berlanga, A., Guiu-Jurado, E., Martinez, S., Porras, J. A., Aragones, G., Sabench, F., Hernandez, M., Aguilar, C., Sirvent, J. J., Del Castillo, D., & Richart, C. (2014). Altered fatty acid metabolism-related gene expression in liver from morbidly obese women with non-alcoholic fatty liver disease. *Int J Mol Sci*, *15*(12), 22173-22187. doi:10.3390/ijms151222173. **PMC4284701**.

9. Ferre, P., & Foufelle, F. (2010). Hepatic steatosis: a role for de novo lipogenesis and the transcription factor SREBP-1c. *Diabetes Obes Metab*, *12 Suppl 2*, 83-92. doi:10.1111/j.1463-1326.2010.01275.x. **PMID: 21029304.**
10. Rong, S., Cortes, V. A., Rashid, S., Anderson, N. N., McDonald, J. G., Liang, G., Moon, Y. A., Hammer, R. E., & Horton, J. D. (2017). Expression of SREBP-1c requires SREBP-2-mediated generation of a sterol ligand for LXR in livers of mice. *Elife*, *6*. doi:10.7554/eLife.25015. **PMC5348127.**
11. Kohjima, M., Enjoji, M., Higuchi, N., Kato, M., Kotoh, K., Yoshimoto, T., Fujino, T., Yada, M., Yada, R., Harada, N., Takayanagi, R., & Nakamuta, M. (2007). Re-evaluation of fatty acid metabolism-related gene expression in nonalcoholic fatty liver disease. *Int J Mol Med*, *20*(3), 351-358. **PMID: 17671740.**
12. Ahmed, M. H., & Byrne, C. D. (2007). Modulation of sterol regulatory element binding proteins (SREBPs) as potential treatments for non-alcoholic fatty liver disease (NAFLD). *Drug Discov Today*, *12*(17-18), 740-747. doi:10.1016/j.drudis.2007.07.009. **PMID: 17826687.**
13. Fujita, K., Nozaki, Y., Wada, K., Yoneda, M., Fujimoto, Y., Fujitake, M., Endo, H., Takahashi, H., Inamori, M., Kobayashi, N., Kirikoshi, H., Kubota, K., Saito, S., & Nakajima, A. (2009). Dysfunctional very-low-density lipoprotein synthesis and release is a key factor in nonalcoholic steatohepatitis pathogenesis. *Hepatology*, *50*(3), 772-780. doi:10.1002/hep.23094. **PMID: 19650159.**
14. Nagaya, T., Tanaka, N., Kimura, T., Kitabatake, H., Fujimori, N., Komatsu, M., Horiuchi, A., Yamaura, T., Umemura, T., Sano, K., Gonzalez, F. J., Aoyama, T., & Tanaka, E. (2015). Mechanism of the development of nonalcoholic steatohepatitis after pancreaticoduodenectomy. *BBA Clin*, *3*, 168-174. doi:10.1016/j.bbacli.2015.02.001. **PMC4661550.**
15. Dorn, C., Riener, M. O., Kirovski, G., Saugspier, M., Steib, K., Weiss, T. S., Gabele, E., Kristiansen, G., Hartmann, A., & Hellerbrand, C. (2010). Expression of fatty acid synthase in nonalcoholic fatty liver disease. *Int J Clin Exp Pathol*, *3*(5), 505-514. **PMC2897101.**
16. Nakamuta, M., Fujino, T., Yada, R., Yada, M., Yasutake, K., Yoshimoto, T., Harada, N., Higuchi, N., Kato, M., Kohjima, M., Taketomi, A., Maehara, Y., Nakashima, M., Kotoh, K., & Enjoji, M. (2009). Impact of cholesterol metabolism and the LXRalpha-SREBP-1c pathway on nonalcoholic fatty liver disease. *Int J Mol Med*, *23*(5), 603-608. doi:10.3892/ijmm_00000170. **PMID: 19360318**
17. Koliaki, C., Szendroedi, J., Kaul, K., Jelenik, T., Nowotny, P., Jankowiak, F., Herder, C., Carstensen, M., Krausch, M., Knoefel, W. T., Schlensak, M., & Roden, M. (2015). Adaptation of hepatic mitochondrial function in humans with non-alcoholic fatty liver is lost in steatohepatitis. *Cell Metab*, *21*(5), 739-746. doi:10.1016/j.cmet.2015.04.004. **PMID: 25955209**

**Chapter II - Contribution of de novo lipogenesis during stages of
nonalcoholic fatty liver disease**

ABSTRACT

The prevalence of nonalcoholic fatty liver disease (NAFLD), characterized by increased liver fat, inflammation, ballooning, and fibrosis, is rapidly growing and this condition has been estimated to affect a quarter of the US population. While some studies have reported altered cholesterol metabolism, increased de novo lipogenesis (DNL) was also been identified as a hallmark feature in individuals with high liver fat. This has led to much activity in drug development and pharmaceutical industries have been developing DNL-inhibiting drugs. Although DNL is a distinguishing characteristic of NAFLD, no studies to date have investigated the contribution of DNL to the progression of the disease. In the present study, patients (n=49) undergoing bariatric surgery were consented at least ten days before surgery to consume deuterated water, and have blood and liver tissue samples obtained on the day of surgery. Patients were separated into three groups according to the extent of liver disease: NAFLD activity score 0-2 (mild), 3-4 (moderate), 5-8 (severe). Liver-TAG and -CE concentrations increased with disease severity ($P<0.001$). The percent of TAG from DNL was higher only in the moderate group ($P<0.001$), but not in the severe group. However, absolute DNL was significantly higher in both moderate and severe groups and was significantly associated with NAFLD characteristics (steatosis, inflammation, ballooning, and fibrosis) and liver enzymes ($P<0.05$ for all). Protein and gene expression analyses revealed that increased liver-CE related due to increased *SOAT2* and decreased *NCEH* activity. Increased FASN activity was associated with decreased fatty acid (FA) oxidation (*CPT1*). For the first time,

these data suggest that DNL promotes NAFLD development and progression by affecting NAFLD characteristics, liver enzymes, and decreasing FA oxidation.

Therefore, DNL inhibition could be a potential treatment for NAFLD patients.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) includes a spectrum of conditions ranging from accumulation of lipids into the liver (steatosis) to inflammation and ballooning, a condition called nonalcoholic steatohepatitis, NASH, and to liver fibrosis and cirrhosis (1-3). The epidemic of NASH and NAFLD continues to grow worldwide and is estimated to affect a quarter of the population of the US and worldwide (4). Recently, NAFLD was predicted to become a leading cause (5) of end-stage liver disease (replacing viral hepatitis (6)) and NASH is now recognized as a major reason for hepatocellular carcinoma-related liver transplantation in the U.S. (7). A wide range of metabolic abnormalities has been associated with NAFLD (1, 8), of which, increased hepatic de novo lipogenesis (DNL) was identified as a key distinguishing characteristic in patients with high liver fat (9, 10). The sources of carbon used for fatty acid synthesis are primarily derived from carbohydrates including glucose and fructose (11-14). In individuals with high liver fat, the Parks lab has shown that ~26% of triacylglycerol (TAG) accumulating in the liver can be derived from DNL (10). Insulin resistance has been shown to play an important role in NAFLD development and has been associated with increased DNL (11, 15). Given the role of insulin in increasing SREBP1 activity (16-18), a master regulator of the DNL and cholesterol pathways, hyperinsulinemia, or insulin resistance have been considered as drivers of DNL in NAFLD patients (11, 15). In contrast, only one study in mice has shown that increased byproducts of DNL (DAG and TAG) decrease insulin action resulting in liver insulin resistance (19). Studies have

also reported altered cholesterol metabolism in NAFLD/NASH patients compared to healthy controls (20). Puri et al reported that liver free cholesterol (FC) was higher in NASH patients compared to healthy individuals (21) and Min et al suggested that increased liver FC in NASH patients was due to increased synthesis, decreased transport, and utilization (20). With regard to DNL, we and others have shown that inhibiting DNL pharmacologically holds the potential to reduce liver fat and cholesterols (22-27). These agents are currently under development as potential treatments for NAFLD and NASH. The role of insulin in the pathogenesis of this disease is complex because the drug-induced reduction in the DNL improved liver fat without changing insulin resistance in some studies (22, 24, 25). However, given that NAFLD is a spectrum of different stages ranging from steatosis to cirrhosis, a major gap in the literature is the understanding of whether DNL increases with worsening disease. The primary hypothesis of this study was that the DNL will be higher as the disease progresses to severe stages. The alternative hypothesis was that the DNL pathway may be higher during the early development of the disease and then plateau during the severe stages of the disease. The data from the present study provides evidence of the role of DNL in the development and progression of NAFLD into more advanced stages. Further, results from this study will aid scientists in the pharmaceutical industry and in academics in understanding whether targeting DNL will be a good strategy at different stages of NAFLD.

METHODS

All methods and procedures were approved by the University of Missouri Institutional Review Board (MU-IRB#2012544), and the study was registered at ClinicalTrials.gov (NCT03683589). Patient recruitment is discussed in the extended methodology section (**chapter II**). Briefly, as shown in **figure 2.1**, 56 patients signed the consent form; 49 patients completed the study and their data were used for analyses.

Patient inclusion criteria and strategy

The inclusion criteria included: men and women (pre and post-menopausal), >22 years of age; overweight/obese with a BMI ≥ 25.9 kg/m²; characteristics of the metabolic syndrome, pre-diabetes (fasting glucose 100-125 mg/dL), or diabetes type II, use of tobacco products or no use of these products; sedentary, ≤ 60 minutes per week of structured physical activity. Exclusion criteria included individuals with acute disease or advanced cardiac, liver, or renal disease; excessive alcohol use; anticoagulation therapy, or any severe co-morbid condition limiting life expectancy < one year; and pre-menopausal women who were pregnant or were trying to become pregnant.

Study design and visits

One (if BMI < 50 kg/m²) or two weeks (if BMI > 50 kg/m²) before surgery, subjects were prescribed a high-protein, liquid diet that was low in carbohydrate as per established guidelines (28-30). Shown in **figure 2.2** is the study design. Once

the patient signed the consent form, they were provided with d₂O for ten days prior to their surgery. The patient consumed 150 ml of d₂O (50 ml doses three times) on day one to increase the d₂O enrichment in the plasma. From day two to ten, patients consumed 50ml per day to maintain d₂O enrichment. On day eleven, two hours prior to the surgery, a sample of the blood was obtained prior to anesthesia, and the FibroScan® 530 Compact (Echosent™ North America, Waltham, MA) was performed to measure liver steatosis, assessed using Controlled Attenuation Parameter (CAP™), and liver stiffness, assessed using Vibration-Controlled Transient Elastography (VCTE™). During surgery, liver tissue (200-300 mg) was collected by the surgeon 30 minutes after induction of anesthesia using a standard wedge biopsy technique (31).

Primary and secondary outcomes

The primary aim of this study was to test the contribution of DNL fatty acids (FAs) during the progression of the disease toward more severe stages. The secondary aim of this study was to test the involvement of cholesterol pathways in disease severity. The exploratory aims were to test the relationship between DNL in TRL and liver, and the relationships between lipogenesis measured objectively with isotopes and indirect measured that are estimates used in past literature. These DNL indices included the ratio of 16:0/18:2 (LD) method, a desaturation index (SCD) - ratio of 16:1/16:0 [SCD₍₁₆₎] and 18:1/18:0 [SCD₍₁₈₎], and an elongation index (EI) - ratio of 18:0/16:0 and 18:1n7/16:1n7.

Blood sample processing and biochemical measurements

Blood samples obtained were immediately processed. An aliquot of the sample was sent to an external laboratory for biochemical testing and the remaining plasma was stored in a -80°C freezer. Biochemical testing was performed as described previously (32). See the extended methodology for more details.

Liver tissue processing and histological scoring

Once the liver tissue was obtained by the surgeon, the sample was immediately transferred to the research lab on ice and was weighted instantly in a 0.9% sterile sodium chloride solution (#306546, BD PosiFlush™, Franklin Lakes, NJ).

Approximately 50 mg of tissue was fixed in ten percent neutral buffer formalin and was stored at 5°C before sending the tissue to a histopathologist for the histological examination. The remaining tissue was frozen and stored at -80°C freezer for other measurements. Liver histological scoring was performed by an experienced hepatopathologist (Dr. Alberto Diaz-Arias). Hematoxylin-eosin and Masson's trichrome staining was performed according to the Brunt scoring scale for NAFLD activity score (NAS) (33) and fibrosis score. Examples of the histologic slides are presented in **figure 2.3**, the criteria are shown in **appendix A**, and the severity of individual NAFLD characteristic in each group is presented in **figure 2.4**.

Western blotting and quantitative real-time PCR

Details regarding technique, antibodies, and PCR primers are presented in the

extended methodology section. Briefly, for western blotting, liver tissue was washed with ice-cold PBS and lysed with a buffer solution. Samples were sonicated, centrifuged, and the supernatant was collected. Total protein content was evaluated using a bovine serum albumin (BSA) concentration assay (BCA) kit. Primary and secondary antibodies were used in 1:1000 and 1:5000 ratio, respectively. Western blots were analyzed via densitometric analysis using ChemiDoc™ MP Imaging System (Image Laboratory Beta 3, Bio-Rad Laboratories, Hercules, CA). Total protein was assessed with Amido black (0.1%, Sigma) to control for the differences in protein loading and transfer (34, 35). Blots were normalized to total protein staining. For RNA extraction, samples were washed with ice-cold PBS and lysed in the buffer, and RNA isolated using the RNeasy mini kit (#74104, Qiagen GmbH, Germany) per the manufacturer's instructions. A cDNA library was synthesized and a Nanodrop spectrometer was used to measure cDNA and RNA purity and assess quality. A list of primers is presented in **table 2.1**. Samples were run on the 7500 Fast Real-Time PCR System (Applied Biosciences, Singapore), and PCR product melt curves were used to assess primer specificity. Data are presented relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the $2^{-\Delta\Delta CT}$ method (34, 36).

Liver-TAG content

Details regarding the liver-TAG assay are provided in the extended methodology section. Briefly, frozen liver samples (approximately 30 mg) were extracted using

the Folch (chloroform and methanol (2:1) mixture) method (37), homogenized for two minutes, and exposed to agitation overnight. One ml of magnesium chloride (4mM) was added, vortexed, and the solution centrifuged for one hour at 1000g at 4°C. The organic phase (500 µL of the bottom layer) was transferred into a new tube, the solvent lipids evaporated, and the pellet reconstituted in tert-butanol and triton-x114 mix (3:2). Total lipids were measured using a commercially available kit (#G7793, Sigma, St. Louis, MO) (35).

Analytical methods

The d₂O was purchased from Cambridge Isotope Laboratory, Inc. (purity ≥99.5%, Catalog#DLM-4-70-0, Andover, MA) and final d₂O enrichments in plasma were measured by cavity ringdown spectroscopy using a Liquid Water Isotope Analyzer with automated injection system, version-2 upgrade (Los Gatos Research, Mountain View, CA) by Metabolic Solutions Inc. (Nashua, NH). This timing of d₂O consumption resulted in a body d₂O enrichment of 0.52±0.14% (mean±SD).

DNL measurements and FA composition in the liver and TRL particle

TRL particles were isolated at 40,000g for 20 h via ultracentrifugation and the top two ml were collected using tube slicing (38). Liver and TRL lipids were isolated using the Folch method (37), TAG-, CE-, and FFA-FAs were separated via TLC, and FAMES were prepared as described previously (38). Fatty acids 14:0, 16:0, and 18:0 made in the DNL pathway was measured using mass isotopomer

distribution analysis (39) on a 6890N gas chromatography coupled to a 5975 mass spectrophotometry detector (Agilent Technologies, Palo Alto, CA). The calculations for DNL using Bederman et al method (40) using N from Patton et al (41) for all three FA are presented in the extended methods and results section. FA composition was measured on a 7890B gas chromatography (Agilent Technologies, Palo Alto, CA). Absolute DNL was calculated by multiplying percent DNL (14:0, 16:0, and 18:0) by total lipid concentration (i.e., percent DNL in 16:0 FA from liver-TAG was multiplied by total TAG concentration) (42). Therefore, the absolute DNL content is presented here as mg/g tissue wet weight (liver lipids) and mg/dL (TRL lipids). Please see the extended methods section for more details.

Patient allocation to groups

Once the liver histologic scoring was obtained from the histopathologist, patients were separated into three groups based on NAS standardized classification (mild: NAS 0-2, moderate: 3-4, and severe: 5-8) (33).

Statistical analysis and calculations

Data are presented as mean \pm SD. One-factor analysis of variance (ANOVA) was performed using the IBM Statistical Package for the Social Sciences (SPSS®, v26, 2019) to test differences between severity groups as described above where NAS classification was used as a between-subject variable. If significance was achieved, Tukey's post-hoc analysis was used to identify differences between

individual groups. Regression analysis was performed using IBM SPSS® (v26, 2019) to test the relationship between percent DNL in the liver-TAG and TRL-TAG, and Bland-Altman plots were created to test the limits of agreement between the two variables. A bivariate Pearson correlation analysis with two-tailed significance was performed using IBM SPSS® (v26, 2019) to test the correlation between other variables. Chi-square analysis was performed to test the percent differences for the metabolic syndrome characteristics. Lastly, for the biochemical measurements, a student t-test was performed using IBM SPSS® (v26, 2019) to test for significance between the pre-surgery data and the data collected on the day of surgery. Liver volume, insulin resistance, NAFLD scores, lipid subclasses, DNL indices, and power calculations are presented in the extended methodology section.

RESULTS

Subject characteristics

As shown in **table 2.2**, subjects had similar age, BMIs, heights, and body weights. As expected, each group, there were more Caucasians and females. The prevalence of metabolic syndrome characteristics was not different between the groups, except for lower HDL which tended to be present in 100% of the subjects in the moderate group (Chi-square $P=0.093$), whereas, in the other two groups the presence of lower HDL was 80% and 78% of the patients, respectively. As expected, steatosis, inflammation, ballooning, and fibrosis scores increased progressively with the severity of the disease ($P\leq 0.002$, for all). As shown in **table 2.3**, results from the FibroScan[®] revealed the highest CAP and VCTE scores in the severe group ($P=0.036$ for both). Lastly, with regard to biomarkers of liver disease, only LFS, APRI, and FAST scores were statistically different between the groups (**table 2.3**) and also correlated positively with the histological scores (NAS, LFS: $r=0.525$, $P<0.001$ APRI: $r=0.364$, $P=0.010$, FAST: $r=0.379$, $P=0.032$, **figures 2.5a-c**).

Analytical and biochemical measurements

As shown in **table 2.4**, deuterium enrichment analyzed in the plasma was not different among groups, suggesting that all subjects consumed the d_2O ($P=0.820$). No differences were observed for plasma glucose concentrations before or on the day of surgery but insulin was significantly higher in the severe group ($P<0.001$). HbA1c was not different among the groups before or on the

day of surgery, however, the weight loss-induced reduction in HbA1c was highest in the moderate group ($P=0.029$). As expected, AST and ALT were significantly different between the groups ($P<0.001$ for both, **table 2.4**). NEFA concentrations were not different among the groups but they correlated positively with AST, BMI, fibrosis score, and AdipoIR, and negatively with the amount of weight loss before surgery (data not shown, results summarized in **figure 2.31a**). No other biochemical variables were different between the groups. A paired t-test analysis between the pre-surgery data and the data collected on the day of surgery showed that AST significantly increased. This increase in AST was expected with rapid weight loss and was transient given the previously published observations (43, 44). Importantly, we collected AST information after four months of bariatric surgery and the AST values were returned to baseline values (**extended figure 2.1**). Plasma glucose, ALP, plasma TAG, cholesterol, and HDL were significantly decreased ($P\leq 0.05$ for all, **table 2.4**).

Changes in liver lipids with the disease severity

As shown in **figure 2.6a**, liver-TAG significantly increased with the severity of the disease ($P<0.001$). The post-hoc analysis revealed that the moderate group's liver-TAG was significantly higher than the mild group ($P<0.05$), and the severe group's liver-TAG was significantly higher than the other two groups ($P<0.05$ for both). Similarly, as shown in **figure 2.6b**, liver-CE was also significantly different between the groups where both moderate and severe groups' liver-CE was significantly higher than the mild group ($P<0.05$ for both). Interestingly, no

differences were observed between the groups for liver-FFA and TRL-TAG (**figure 2.6c-d**). Further, individuals whose plasma TAG concentrations were highest before surgery were the ones whose TAG reduced the most while on a pre-surgery diet (**figure 2.6e**). Additionally, a strong relationship was observed between liver-TAG and liver-CE (**figure 2.6f**). As shown in **figure 2.7**, an increase in liver-TAG was strongly associated with the increase in histological grading of steatosis (**figure 2.7a**), inflammation (**figure 2.7b**), ballooning (**figure 2.7c**), and fibrosis (**figure 2.7d**). Additionally, liver-TAG also correlated significantly with both AST (**figure 2.7e**) and ALT (**figure 2.7f**). Similarly, an increase in liver-CE with the severity was also associated with the histological grading of steatosis (**figure 2.7g**), inflammation (**figure 2.7h**), ballooning (**figure 2.7i**), and fibrosis (**figure 2.7j**). Lastly, liver-CE also correlated with both AST (**figure 2.7k**) and ALT (**figure 2.7l**).

DNL in the liver - relationship with the disease severity

DNL measured in the liver-TAG was variable between the groups: As shown in **figure 2.8a**, the fractional de novo lipogenesis measured in liver-TAG 16:0 was significantly different between the groups ($P < 0.001$). The average percent DNL in the mild group was $6.0 \pm 3.0\%$ whereas in the moderate group's percent DNL was $14.3 \pm 6.2\%$ which was significantly higher than the mild group ($P < 0.05$). Surprisingly, in the group with severe disease, percent DNL was $4.7 \pm 2.1\%$ which was significantly lower than the moderate group ($P < 0.05$) but was not different compared to the mild group. However, the absolute DNL was significantly

different between groups ($P=0.005$) with the highest's been in the moderate and severe groups compared to the mild group ($P\leq 0.001$ for both), and no differences were observed between the moderate and the severe group (**figure 2.8b**, mild: 0.6 ± 0.7 mg/g tissue wet weight, moderate: 5.6 ± 3.7 mg/g wet weight, severe: 4.9 ± 3.6 mg/g wet weight). Further, to identify DNL at each stage of the NAFLD (i.e., steatosis to fibrosis), patients were categorized based on their disease stage. Interestingly, DNL increased progressively from steatosis to inflammation, and ballooning but was not different in patients who had fibrosis ($P=0.039$, **figure 2.8c**). Lastly, as shown in **figure 2.8d**, when patients were compared based on the presence (NAFLD) or absence (healthy) of NAFLD characteristics, DNL was significantly higher in the NAFLD group (liver-DNL $P=0.043$, TRL-DNL $P=0.092$).

DNL measured in the liver-CE was increased with the severity of the disease but not altered in the liver-FFA: As shown in **figure 2.9a**, the percent DNL measured in the liver-CE 16:0 was significantly different between the groups ($P=0.048$) with the highest being found in the moderate group ($P<0.05$). Percent DNL for liver-CE was not statistically different in the severe group compared to the mild or the moderate group. Further, as shown in **figure 2.9b**, absolute DNL in liver-CE exhibited the same pattern as TAG ($P=0.001$). Both the moderate and severe groups were significantly higher compared to the mild group ($P<0.05$ for both). Lastly, as shown in **figures 2.9c-d**, no differences were observed for DNL measured in the liver-FFA 16:0 (both percent and absolute).

Liver DNL correlated with the NAFLD characteristics: As shown in **figures**

2.10a-d, absolute DNL measured in the liver-TAG strongly correlated with the histological grading of steatosis (**figure 2.10a**), inflammation (**figure 2.10b**), ballooning (**figure 2.10c**), and fibrosis (**figure 2.10d**). Additionally, absolute DNL 16:0 measured in the liver-TAG also correlated with both AST (**figure 2.10e**) and ALT (**figure 2.10f**). These relationships are summarized in **figure 2.31a**. Similar results were observed for the absolute DNL measured in the liver-CE 16:0 as well i.e., a strong relationship with histological grading of steatosis (**figure 2.10g**), inflammation (**figure 2.10h**), ballooning (**figure 2.10i**), and fibrosis (**2.10j**). Lastly, absolute DNL in the liver-CE 16:0 also correlated with both AST (**figure 2.10k**) and ALT (**figure 2.10l**).

DNL in plasma TRL - relationship with liver-DNL

DNL in TRL-TAG was variable between the stages: As shown in **figure 2.11a**, the percent DNL measured in the TAG 16:0 in TRL particles reflected results similar to that of liver-TAG percent DNL i.e., significantly higher in the moderate group compared to the mild group and the severe group ($P < 0.05$ for both).

However, no differences were observed between the groups for the absolute DNL in TRL-TAG (**figure 2.11b**).

DNL was not altered in plasma TRL-CE: As shown in **figure 2.12a**, the percent DNL measured in the TRL-CE was not different between the groups.

DNL relationship between TRL-TAG and liver-TAG: As shown in **figure 2.13a**, regression analysis revealed a statistically significant and positive relationship

between the percent DNL measured in the liver-TAG and TRL-TAG ($r=0.747$, $P<0.001$, equation: liver-TAG %DNL = $0.007+0.918 \times$ TRL-TAG %DNL). **Figure 2.13b** represents a Bland-Altman plot between the Ln_mean and Ln_difference of the percent DNL in liver-TAG and TRL-TAG. A non-significant bias ($P=0.945$) and coefficient ($P=0.056$) were observed with the majority of patients falling within the limit of agreement boundaries.

DNL enzymes and their relationship with isotopic DNL

mRNA expression of the key DNL genes: As shown in **figure 2.14**, the mRNA expression of the *SREBF1* was not different between groups, however, *ChREBP* appeared to follow the trend similar to the percent DNL measured in the liver-TAG and TRL-TAG (**figures 2.8a & 2.11a**, respectively). No changes were observed in *ACC* or *INSIG1* mRNA, but *FASN* and *SCD* mRNA increased progressively with the severity of the disease. Further, both the *FASN* and *SCD* expression tended to be higher in the moderate group ($P=0.07$ for both) and were significantly higher in the severe group compared to the mild group ($P<0.05$ for both, **figure 2.14**).

Protein expression of the key DNL enzymes: As shown in **figures 2.15a-b**, no significant changes were found in the protein expression of SREBP, ACC, pACC, and pACC/ACC ratio, an indicator of ACC activation. However, only FASN protein was significantly different between the groups ($P=0.036$) with the highest expression in the moderate group compared to the mild group ($P<0.05$).

However, no differences were seen for FASN in the severe group - a trend similar to the percent DNL measured in the liver-TAG and TRL-TAG (**figures 2.8a & 2.11a**, respectively). The expression of SCD appeared to follow a similar trend ($P=0.064$), and no differences were observed for INSIG1 protein.

Relationship of DNL enzymes with lipogenic fatty acids measured directly in the liver: As shown in **figures 2.16a-b**, FASN protein expression significantly and positively correlated with both the percent DNL and absolute DNL.

Relationship with the DNL measured in the TRL particle: As shown in **figures 2.17a-d**, DNL measured in the TRL-TAG significantly and positively correlated with SREBP1 ($r=0.378$, $P=0.021$), FASN ($r=0.655$, $P<0.001$), and SCD1 ($r=0.518$, $P=0.001$) and negatively correlated with DGAT2 ($r=-0.333$, $P=0.038$). Furthermore, as shown in **figures 2.18a-b**, FASN protein expression also correlated significantly and positively with the absolute DNL in the TRL-TAG ($r=0.677$, $P<0.001$) and percent DNL in the TRL-CE ($r=0.481$, $P=0.003$).

Protein and mRNA expression of other enzymes and their relationship to the disease severity

For enzymes involved in the TAG metabolism (**figures 2.19a-c**), the mRNA expression of *DGAT1* increased significantly ($P=0.005$) and *DGAT2* appeared to increase with the severity although their protein expressions were not different. In contrast, *APOB* mRNA expression was not different between the groups but

the protein expression was higher in the moderate group (APOB100, $P=0.109$). For enzymes involved in FA transport (**figures 2.20a-b**), *CD36* mRNA expression increased significantly ($P<0.001$) with the highest being found in the severe group compared to both mild and the moderate groups ($P<0.05$ for both). However, *FABP1* was not different and *FATP5* tended to be reduced with increasing severity ($P=0.061$). With regard to FA oxidation enzymes, *CPT1* mRNA expression was not different between groups ($P=0.133$) but the *PPAR- α* reduced both in the moderate and the severe groups ($P=0.002$), and *PGC1- α* reduced significantly in the severe group ($P=0.049$). Further, *CPT1* mRNA expression was negatively associated with FASN protein expression ($r=-0.543$, $P=0.005$, **figure 2.20c**) and the percent DNL in liver-TAG ($r=-0.405$, $P=0.014$, **figure 2.20d**). For enzymes involved in cholesterol metabolism (**figure 2.21**), *SREBF2*, a master regulator of cholesterol synthesis pathway tended to change with the severity ($P=0.06$). The mRNA expression of *ACAT2* (an indicator for ketolysis) was not significantly different between groups ($P=0.157$), but *HMGCR* expression was significantly higher in the moderate group ($P=0.015$). The expression of *ABCG8* (cholesterol transporter) and *LDL-R* (cholesterol uptake) expression were not different. However, *SOAT2* (cholesterol esterification enzyme also referred to as *ACAT2* in the previous literature) expression tended to increase in the moderate group compared to the mild group ($P=0.10$) and was significantly higher in the severe group compared to the mild group ($P<0.05$). *NCEH* expression (cholesterol ester hydrolase enzyme) was significantly lower in both the moderate and the severe groups compared to the mild group (ANOVA

$P=0.008$, group difference $P<0.05$ for both). These findings are summarized in **figure 2.31b**. Lastly, as shown in **figures 2.22a-b**, the individuals with higher steatosis grades tended to have higher expressions of the FASN protein ($r=0.306$, $P=0.065$) and significantly lower expressions of the CD36 protein ($r=-0.416$, $P=0.009$).

DNL measurement in FA species other than 16:0 FA

Shown in **figures 2.23a-j** is the percent DNL measured in the FA 14:0 and FA 18:0 in the liver-TAG, liver-CE, liver-FFA, TRL-TAG, and TRL-CE. Only percent DNL measured in the liver-TAG 18:0 tended to be different between the groups ($P=0.052$) with the highest being in the moderate group ($P<0.05$, **figure 2.23f**). Similar to the absolute DNL measured in the liver-TAG 16:0 and liver-CE 16:0 (**figures 2.8b and 2.11b**), significant differences were observed between the groups for the absolute DNL measured in the liver-TAG 14:0 ($P=0.004$, **figure 2.24a**), liver-TAG 18:0 ($P<0.001$, **figure 2.24e**), and liver-CE 18:0 ($P=0.008$, **figure 2.24f**). For liver-CE 14:0, absolute DNL tended to increase with the severity ($P=0.054$, **figure 2.24b**). Post-hoc analysis revealed that for the measurement performed in the liver-TAG 14:0, liver-TAG 18:0, and liver-CE 18:0, absolute DNL was significantly higher in the moderate group and the severe group compared to the mild group ($P<0.05$ for all, **figure 2.24a, e-f**, respectively).

DNL-FAs contribution to the lipid stores with the disease severity

As shown in **figure 2.25**, with the increase in the severity of the disease, total

absolute DNL was significantly different between groups ($P<0.001$) with the highest contribution of DNL in the moderate and the severe groups compared to the mild group ($P<0.05$ for both). Interestingly, as shown in **figure 2.26**, compared to the non-DNL sources of FA (unlabeled), the direct contribution of DNL-FA in the liver lipid pool appeared to be low in both liver-TAG and liver-CE fractions.

Role of insulin resistance

As shown in **figure 2.27**, calculators of the insulin resistance predicted the severity of the disease. HOMA-IR was significantly different between the groups with the highest being in the severe group (ANOVA $P<0.001$, group effect $P<0.05$, **figure 2.27a**). Similarly, an index of insulin sensitivity (QUICKI) was also significantly different between the groups ($P<0.001$, **figure 2.27b**). Further, individuals in the moderate group had significantly lower insulin sensitivity compared to the mild group ($P<0.05$) and the severe group's insulin sensitivity was significantly lower than both the mild and the moderate group ($P<0.05$ for both, **figure 2.27b**). Lastly, AdipoIR was significantly different between the groups with the highest being in the severe group (ANOVA $P<0.001$, group effect $P<0.05$, **figure 2.27c**). Furthermore, insulin sensitivity calculated by QUICKI also significantly correlated with the absolute DNL in both the liver-TAG and the TRL-TAG (liver-TAG: $r=-0.315$, $P=0.029$, TRL-TAG: $r=-0.430$, $P=0.002$, **figures 2.28a-b, figure 2.31a**), and the protein expression of the key DNL enzymes (SREBP1: $r=-0.331$, $P=0.046$, FASN: $r=-0.330$, $P=0.046$, **figures 2.28c-d**). In

other words, the greater the use of peripheral glucose utilization, the lower the insulin concentration and this affects liver gene expression.

Changes in FA species with the disease severity

Presented in **figure 2.29** was the FA composition of different liver and TRL lipids. In liver-TAG, percent areas of 14:0, 16:0, 16:1n7, 18:1n9 increased whereas 18:2n6, 20:3n6, 22:4n6, and 22:6n3 decreased significantly ($P<0.05$ for all). In liver-CE, percent areas of 16:1n7 and 18:2n6 increased whereas 18:0, 20:5n3, and 22:5n6 decreased significantly ($P<0.05$ for all). No changes were observed for FA composition in liver-FFA. While the percent areas of 18:1n7 increased, and 18:2n6 decreased significantly in TRL-TAG ($P<0.05$ for both), no changes were observed in TRL-CE FA composition. As shown in **table 2.5**, similar to the total liver-TAG, the absolute concentrations of all the subclass (SFA, MUFA, PUFA, omega-3 FAs, omega-6 FAs, essential FAs, and non-essential FAs) but one (EPA+DHA) in the liver-TAG increased significantly with the severity of the disease ($P<0.001$ for all). However, when the percentages of each subclass were calculated, the percent of SFA increased progressively with the disease (ANOVA $P<0.001$, $P\leq 0.05$ for between groups), percent MUFA was significantly higher in the moderate group and the severe group ($P\leq 0.05$) compared to the mild group. Percent PUFA, percent omega-3 FAs, percent omega-6 FAs, and the percent of EPA+DHA, all significantly and progressively reduced with the severity of the disease (ANOVA $P<0.001$ for all, $P\leq 0.05$ for between groups). Lastly, the ratio of omega-6/omega-3 increased with the disease severity

($P < 0.001$) and was significantly higher in the moderate and the severe group compared to the mild group ($P < 0.05$ for both). Interestingly, a significant relationship was also observed between the omega-6/omega-3 ratio and NAFLD activity score ($r = 0.608$, $P < 0.001$, **figure 2.30a**). As shown in **figure 2.30b**, a significant relationship was observed between percent DNL and percent MUFA ($r = 0.366$, $P = 0.010$). The protein and mRNA expression of *FASN* and *SCD* were also found to be related to each other (protein: $r = 0.329$, $P = 0.058$, **figure 2.30c**, mRNA: $r = 0.679$, $P < 0.001$, **figure 2.30d**). Subclass analysis of the liver-CE revealed a significant difference in the concentration of the SFA, MUFA, PUFA, omega-6 FAs, essential FAs, and non-essential FAs (ANOVA $P \leq 0.001$ for all). For SFA, MUFA, PUFA, essential FAs, and non-essential FAs, the concentrations were significantly higher in the moderate and the severe groups compared to the mild group ($P \leq 0.05$) whereas omega-6 FAs concentration was higher only in the severe group compared to the mild group ($P \leq 0.05$). No differences were observed in the percent of each subclass, except for percent EPA+DHA ($P = 0.008$) which was significantly lower in the severe group compared to the mild group ($P \leq 0.05$). No differences were observed in the subclass analysis of the liver-FAA whereas, in the TRL-TAG, the percent PUFA ($P = 0.001$) and omega-6 FAs ($P = 0.002$) were significantly different. Compared to the mild group, both the moderate and the severe groups exhibited a significantly higher percent of PUFA and omega-6 FAs ($P \leq 0.05$ for both) in the TRL-TAG.

DNL indices, relationship with the disease severity, and comparison with isotopic DNL

DNL indices and group comparisons: The area ratio of 16:0/18:2 was found to be significantly different only in the liver-TAG ($P < 0.001$, **table 2.6**) where the score was significantly higher in the moderate group compared to the mild group ($P \leq 0.05$) and significantly higher in the severe group compared to the mild and the moderate groups ($P \leq 0.05$). The area ratio of 16:1n7/16:0 (SCD₍₁₆₎ index), 18:1n9/18:0 (SCD₍₁₈₎ index), and 18:0/16:0 (elongation index) were significantly different when calculated in the liver-CE ($P < 0.001$ for all) and SCD₍₁₆₎ index in the TRL-CE ($P = 0.038$). For the SCD₍₁₆₎ index in the liver-CE, only the severe group had a significantly higher area ratio compared to the mild and the moderate groups ($P \leq 0.05$ for both) whereas, in the TRL-CE, only the severe group's area ratio was significantly higher than the mild group ($P \leq 0.05$). The SCD₍₁₈₎ index of the liver-CE increased progressively with the disease i.e., the moderate group's ratio was significantly higher than the mild group ($P \leq 0.05$) and the severe group's ratio was significantly higher than both the mild and the moderate groups ($P \leq 0.05$ for both). The elongation index (18:0/16:0) in the liver-CE was significantly higher in both the moderate and the severe groups compared to the mild group ($P \leq 0.05$ for both) and the elongation index (18:1n7/16:1n7) was not different between the groups.

DNL indices relationships with NAFLD activity score: As shown in **table 2.7**, a significant relationship between the NAFLD activity score and LD score

calculated in the liver-TAG ($r=0.660$, $P<0.001$) and liver-CE ($r=-0.372$, $P=0.009$). For the SCD₍₁₆₎ index, a relationship with NAFLD activity score was observed in the liver-CE ($r=0.480$, $P<0.001$) and TRL-CE ($r=0.324$, $P=0.026$) whereas for the SCD₍₁₈₎ index, a significant relationship was observed in liver-CE ($r=0.576$, $P<0.001$) and TRL-TAG ($r=0.298$, $P=0.038$). The elongation index (18:0/16:0) correlated with the liver-CE ($r=-0.489$, $P<0.001$). Lastly, for the elongation index (18:1n7/16:1n7), a statistically significant and negative relationship was observed with the liver-CE and TRL-TAG ($r=-0.290$, $P=0.043$; $r=-0.307$, $P=0.032$, respectively).

Comparison of isotopic DNL and DNL indices: As shown in **table 2.7**, the LD scores significantly correlated with the absolute DNL measured in the liver-TAG ($r=0.514$, $P<0.001$). For the SCD₍₁₆₎ index and the SCD₍₁₈₎ index, the calculated scores significantly correlated with the absolute DNL in the liver-CE (SCD₍₁₆₎ index: $r=0.318$, $P=0.026$, SCD₍₁₈₎ index: $r=0.608$, $P<0.001$) and the liver-FFA (SCD₍₁₆₎ index: $r=0.393$, $P=0.005$, SCD₍₁₈₎ index: $r=0.441$, $P=0.002$). Lastly, the elongation index (18:0/16:0) scores significantly correlated with both the percent and absolute DNL in the liver-CE (percent DNL: $r=-0.420$, $P=0.003$ absolute DNL: $r=-0.553$, $P<0.001$, respectively) and the elongation index (18:1n7/16:1n7) scores significantly correlated with the absolute DNL in the liver-TAG ($r=-0.306$, $P=0.037$) and TRL-TAG ($r=-0.302$, $P=0.035$).

DISCUSSION

Increased DNL is a key distinguishing characteristic indicating the presence of NAFLD yet no study to date has determined whether graded increases in DNL occur as the disease progress (9). The present study provides evidence that the FAs made from the DNL pathway contribute to the development and progression of the disease by impacting each histological characteristic of NAFLD (i.e., steatosis, inflammation, hepatocellular ballooning, and fibrosis) and liver enzymes (AST and ALT) in the presence of insulin resistance. As shown in **figure 2.31a**, DNL correlated positively with liver-TAG, NAFLD characteristics, liver enzymes, and AdipoIR and negatively correlated with insulin sensitivity, whereas NEFA concentrations were associated with BMI, weight loss, fibrosis score, and AdipoIR. While the use of human tissues in this study did not permit mechanistic studies to test the precise role of DNL-FAs at each stage of the disease progression, these data provide a detailed snapshot of the contribution of DNL to the development and progression of NAFLD.

Increased TAG and CE in the liver but not in TRL were associated with the grade of NAFLD characteristics

Increased liver lipids with the disease severity: With regard to lipid measurements in the liver, as expected and consistent with the previous literature, the liver-TAG content was increased with the disease severity and liver-FFA was not altered (21). Puri et al conducted a lipidomic analysis of NAFLD and NASH patients' liver tissue and compared it to healthy individuals.

Liver-TAG was significantly higher in NAFLD and NASH patients compared to healthy individuals and no differences were reported in liver-FFA. However, inconsistent with the previous studies, liver-CE increased with the severity of the disease (20, 21). In the present study, these results were further supported by the increased activity of *SOAT2* (cholesterol esterification enzyme) and decreased activity of *NCEH* (cholesterol hydrolase enzyme, **figure 2.21**). The outcomes in the present study and the past studies were opposite (20, 21). This may be due to the differences observed between the study populations. In the studies conducted by Puri et al and Min et al, patients' BMI was around 35 kg/m² whereas the BMI of patients in the present study was around 46 kg/m². The activity of *SOAT2* can be increased by leptin (45), a hormone found in higher concentrations in individuals with greater BMI (46). Given the patients in the present study were recruited from the bariatric clinic, the leptin levels were likely higher in these patients (47) compared to the previous study (20) which may have resulted in a higher *SOAT2* activity with the disease severity as shown by Alger et al in mice (48). Furthermore, in the present study, we also observed a decreased *NCEH* activity with the increased severity whereas Min et al reported the opposite (i.e., higher in NASH patients) (20). Overall, the increases observed in liver-CE in the moderate group were consistent with gene signatures of increased synthesis, esterification, and decreased cholesterol hydrolase activity (**figures 2.21 & 2.31b**). Further increased liver-CE in the severe group could be attributed to higher esterification of free cholesterol by *SOAT2* and a significant decrease in the *NCEH* activity (**figure 2.21 & 2.31b**).

Inefficient TRL secretion with the disease severity: When lipids were measured in TRL particles, no changes were observed in the total TRL-TAG concentrations between the groups. This is somewhat surprising since previous studies conducted in patients with high liver fat exhibited higher VLDL-TAG concentrations and secretion rates compared to individuals with low liver fat (9, 49). However, both studies included individuals with an average BMI of 35 kg/m² whereas in the present study average BMI was around 45 kg/m². Similar to the present result, Lytle et al (50) conducted a study in weight-stable patients with an average BMI of 47 kg/m² undergoing bariatric surgery and reported no differences in VLDL-TAG concentrations between the patients with low (3±1%) vs high liver fat (11±4%). Moreover, they also reported that the VLDL concentrations were higher in both groups (800-900µmol/L which was around 75-80 mg/dL) (50) compared to what was observed previously in non-obese and class I obese subjects which were around 400µmol/L (51). In the present study, we isolated TRL-TAG (which was a combination of chylomicrons and VLDL particles) and because patients were fasting, this fraction primarily contains VLDL (10, 38). The average TRL-TAG concentration of the patients from the present study was 78±50 mg/dL which was consistent with the study conducted by Lytle et al. However, these results are not fully comparable because unlike the patients from the Lytle et al study (50), patients in the present study were not weight-stable and were required to lose weight before the surgery. Patients were on a pre-surgery, low-calorie, liquid diet (600-1000 kcals/day) for one to two weeks resulting in negative energy balance. As a result, these patients lost

4±3% (all subjects' average) of their body weight and reduced plasma TAG by 17±26% (all subjects' average). Further, the individuals with the highest pre-surgery plasma TAG concentrations reduced their plasma TAG the most while on the liquid diet ($r=0.796$, $P<0.001$, **figure 2.6e**). This may have resulted in similar plasma TAG concentrations across the groups on the day of surgery and therefore no differences were observed in their TRL-TAG concentrations. Further, Lytle et al reported that individuals with low liver fat exhibited a positive relationship between the liver fat and VLDL secretion rates - a finding consistent with Fabbrini et al (49). However, Lytle et al also reported a negative relationship between liver fat and VLDL secretion rates in individuals with high liver fat (50). Interestingly, hepatic palmitate uptake, as measured by infusing an intravenous bolus of [9,10-³H]palmitate 30 minutes before biopsy (a measure of plasma FA uptake in the liver), correlated positively with the liver fat in the group with low liver fat but not in the group with high liver fat. Based on these outcomes Lytle et al concluded that defective VLDL secretion, rather than an increased FA uptake in the liver, contributed to increases in the liver fat (50). Indeed, in the present study, the expression of CD36 protein (a direct marker of FA uptake in the liver) was not different between the groups, and the mRNA expression of *FABP1* and *FATP5* tended to be lower with severity (**figure 2.20a-b**). Our data suggest that the total NEFA flux to the liver was not different, and because the liver fat increased with the severity, similar TRL-TAG concentration across groups in the present study was perhaps due to defect in TRL secretion. However, in the present study, the protein expression of APOB100 tended to be higher in the

moderate group but not in the other two groups ($P=0.109$) suggesting there may be more smaller-sized particles being secreted in the moderate group - a finding consistent with Lytle et al (50).

Liver-TAG and liver-CE were associated with NAFLD characteristics: Results from the correlational analysis revealed that liver-TAG (a direct measure of liver fat) showed a strong relationship with all the histological characteristics of NAFLD (steatosis, inflammation, ballooning, and fibrosis, **figures 2.7a-d**) and both liver enzymes (AST and ALT, **figures 2.7e-f**). These findings were similar to results reported by Chalasani et al who utilized steatosis score as an indicator of liver fat (52). Chalasani et al reported that increasing levels of liver fat were positively and significantly associated with lobular inflammation and zone-three fibrosis whereas the accumulation of fat in a specific location (pan-acinar steatosis) was positively associated with ballooning and advanced fibrosis. By contrast, Brill et al utilized MRS to calculate the liver fat volume and divided their study participants into ten quantiles based on the liver fat content (53). None of these patients appeared to have higher grades of inflammation, ballooning, or fibrosis, whereas in the present study, we included patients across each grade for steatosis (ranging from 0-3), inflammation (0-2), ballooning (0-2), and fibrosis (0-4). Although patients with high liver fat may not necessarily have inflammation, ballooning, or fibrosis (53), individual data from the present study showed that all patients with any degree of inflammation, ballooning, or fibrosis also had steatosis suggesting steatosis that is a key early contributor to the

development and progression of NAFLD.

In addition to liver-TAG, we also observed similar results with liver-CE (**figures 2.7g-l**). However, limited data were available in the literature on the liver-CE contribution in NAFLD progression. An abstract presented at the American Gastroenterology Association meeting by Shores et al reported a direct correlation between liver-TAG and liver-CE (54), a finding consistent with the present study (**figure 2.6f**). Overall, findings from this study and other literature suggest that both liver-TAG and liver-CE stores may contribute directly to the development and the progression of NAFLD (**figures 2.7g-l**).

DNL contribution in NAFLD severity

DNL was associated with the degree of severity of NAFLD characteristics: As discussed already, increased liver lipids are not likely due to increased NEFA flux but may be due to defect in TRL secretion, as evidenced by unchanged CD36 protein expression and TRL concentrations between the groups. These data suggest that the increased liver lipids were either due to increased endogenous synthesis (DNL) or reduced oxidation of FAs. With regard to DNL measurements in the liver, the FAs made from the DNL pathway directly contributed a small portion to the total lipid stores in the liver (**figure 2.25**), however, patients in the present study were under negative energy balance and exhibited relatively lower percent DNL compared to previously-published data (9, 10, 15, 55-58).

Nonetheless, the quantity of DNL-FAs was related to many characteristics of NAFLD (**figures 2.10a-f, figure 2.31a**). Given the extent of fasting, it is likely the

values of DNL are at the low physiologic levels.

As observed previously, increases in the secretion rates (49) of VLDL-TAG were attributed to the higher contribution of FA from the non-systemic sources (49). Importantly, to differentiate NAFLD patients from control subjects, previous studies have used liver-TAG, instead of biopsy-proven histological scores, which limits the ability to identify a severe group. Therefore, these studies were also unable to distinguish the direct contribution of DNL-FAs at different stages of the disease. In the present study, biopsy-proven histological scores were utilized to identify the severity of the disease. A high percent DNL measured (both in liver-TAG and TRL-TAG) in the moderate group supports DNL-FA's role in the early development of NAFLD. However, the appearance of a lower percent DNL in the severe group (for both liver-TAG and TRL-TAG) was merely due to high TAG content in the liver and the TRL particle. Indeed, when the absolute DNL was calculated, the final contribution of FAs from the DNL in the severe group was similar to what was observed for the moderate group, suggesting DNL contributed not only during the early development of NAFLD but also during the progression of the NAFLD into more severe stages. One study conducted by Lawitz et al (55), presented in abstract form at the AASLD 2017 meeting reported no changes in the percent DNL with the disease severity. However, these investigators used stiffness, as measured by magnetic resonance elastography (MRE) scan, as an indicator for the severity of NASH (stiffness: mild < 3.64 vs severe ≥ 3.64 kPa). As mentioned earlier, data in the present study were clustered based on histological scoring and we found significantly lower percent

DNL in the severe group compared to the moderate group (both in liver-TAG and TRL-TAG). However, when we clustered all of our patients by the stages observed in the typical progression of NAFLD pathogenesis (steatosis, inflammation, ballooning, and fibrosis, **figure 2.7c**), we observed a linear increase in DNL, except in patients with fibrosis. Another study conducted by Smith et al in lean and obese individuals with or without NAFLD reported a significantly higher percent DNL in patients with NAFLD compared to lean and obese individuals without NAFLD (15). Consistent with these findings (9, 15), we observed similar results in our group when we clustered our patients in groups similar to the previous study (healthy - no histological abnormalities, NAFLD - the presence of disease) i.e., a higher percent DNL for both liver-TAG ($P=0.043$) and TRL-TAG ($P=0.092$) in the NAFLD group compared to healthy group (**figure 2.8d**). On a side note, as shown in **figure 2.13**, a strong relationship observed between liver-TAG and TRL-TAG confirms the previous findings, but in a larger sample size, that the DNL measured in the TRL-TAG was reflective of DNL measured in the liver-TAG suggesting future studies can continue to use TRL-DNL as an indicator of liver DNL (10).

Changes observed in isotopic DNL were consistent with changes observed at the molecular level: The results presented above were also supported by the expression of mRNA and proteins involved in the DNL pathway. No significant changes were observed in mRNA expression for most of the genes, and this was likely due to high variability which is somewhat expected in human samples

(**figure 2.14**). However, no changes in *SREBF1* and a significant increase in the mRNA expression of *FASN*, *SCD*, and *DGAT1* (**figures 2.14 and 2.19**) with the disease severity was likely a direct effect of higher insulin concentrations (independent of *SREBP1* activation) (17). These results are consistent with the previous studies that reported significant increases in the expression of *SREBP1*, *ACC*, *FASN*, and *SCD* in NAFLD and NASH (59-62). However, changes observed in the mRNA expression were not well translated into protein expression for some enzymes. The protein expression for *FASN* and *SCD* followed a trend similar to what was observed for actual DNL measured isotopically i.e. *FASN* and *SCD* activities increased in the moderate group but were not different in the severe group (60). Similar findings were also observed by Dorn et al who reported increased *FASN* activity in patients with steatosis but not in NASH patients (61). Decrease *FASN* activity in the severe group has been attributed to increased ROS production (63, 64) which may inactivate *FASN* protein through AMPK pathway activity (65). Moreover, strong relationships were observed between liver-TAG DNL and *FASN* protein (**figure 2.16**), between TRL-TAG DNL and *SREBP1*, *FASN*, and *SCD1* (**figure 2.17**), and between TRL-CE DNL and *FASN* protein expression (**figure 2.18**) suggest that changes observed in isotopic measured DNL with the severity were due to changes observed in the activity of DNL enzymes at the molecular level (59).

DNL-FAs are used for TAG synthesis and cholesterol esterification: For the first time, we measured DNL in liver-CE, liver-FFA, and TRL-CE. However, only in

liver-CE, DNL was increased in the moderate group but not in the severe group - similar to what was observed in liver-TAG (**figure 2.9**). These data suggest that DNL-FAs are not only utilized in TAG synthesis (10) but are also utilized for cholesterol esterification and storage (**figure 2.25**). Further, these changes were strongly linked with disease progression as represented by a positive relationship observed between liver-CE DNL and histological characteristics of NAFLD (**figures 2.10g-l**).

DNL-FAs are driven toward storage and related to reduced FA oxidation in NAFLD: With regard to the handling of DNL-FAs, previous studies have shown that DGAT2 acts upstream of DGAT1, primarily utilizing DNL-FAs, and promoting lipid storage in HepG2 cells whereas DGAT1 primarily utilizes NEFA and incorporates the majority of FAs into the VLDL particles which are secreted out into the circulation (66, 67). Here, a negative relationship was observed between the percent DNL in the TRL particle and *DGAT2* activity suggesting that DNL-FAs were stored more (or secreted less through TRL particle) when *DGAT2* activity was high (**figure 2.17d**). Further, FASN protein activity was positively associated with steatosis grade (**figure 2.22a**). These data and previous studies point to a unique mechanism by which DNL-FAs lead to hepatic lipid accumulation (67-69). While increased mRNA expression for both *DGAT1* and *DGAT2* were perhaps due to higher concentrations of plasma glucose and insulin (activators of DGAT isoforms) (70, 71), as explained by Jung et al and Liu et al, the lack of changes observed in the expression of both proteins were likely

because of the higher degradation/inactivation of proteins due to increased ROS (72, 73). With regard to NEFA contribution, CD36 expression correlated negatively with steatosis grade (**figure 2.22b**) - this finding was unexpected and was not consistent with the previous literature in which liver CD36 expression was higher in bariatric patients with NAFLD/NASH characteristics (74, 75). These differences could be due to pre-surgery weight loss requirements that may have reduced CD36 expression in our patients (76, 77). These data suggest that the secretion of TRL and storage capacity of lipids were not different with severity, additionally, because DNL-FA contributed to a small portion of the total liver lipid stores, and NEFA did not contribute to the steatosis grade, the only possible explanation for the increased liver stores was perhaps due to decreased FA oxidation. In this study, we measured the mRNA expression of *CPT1* (mitochondrial FA uptake gene, inhibited by DNL byproduct, malonyl-CoA), *PPAR- α* (promoter of β -oxidation), and *PCG1- α* (activator of mitochondrial biogenesis). Interestingly, consistent with previous research, both *PPAR- α* and *PGC1- α* decreased with the disease severity suggesting FA oxidation becomes reduced as the disease progresses (64). These data were also confirmed in a larger cohort by our group (Moore MP et al, unpublished work). Further, because an intermediate of DNL, malonyl-CoA, inhibits *CPT1* (78, 79), we performed a correlation analysis to determine if the reduction in FA oxidation was due to increased DNL and indeed we found a negative relationship between FASN protein expression and *CPT1* mRNA expression, and the percent DNL in the liver-TAG and *CPT1* mRNA expression (**figure 2.20c-d**). These data suggest

that the reduction in FA oxidation with the severity of the disease was indirectly due to increased lipogenesis (78, 79).

DNL in 14:0 and 18:0 contributed minimally to total absolute DNL: In the past, DNL was measured primarily in 16:0 FA because 16:0 was the primary product of FA synthesis (a major SFA accounted in the total FA pool) and was considered as a surrogate marker of total DNL (9, 10, 42, 80-82). We quantitated DNL-FAs in FA species other than 16:0. The detection of excess M1 in 14:0 and 18:0 suggests that these FA can also be synthesized through the DNL pathway (83). Newly-made 14:0 results from an increased methyl group (acetyl-CoA) crowding on the KS binding channel of the FASN enzyme resulting in premature termination of synthesis and 18:0 is a normal product of FASN enzyme (synthesizes minor amounts of 18:0) (84, 85). In the present study in liver-TAG, a trend similar to 16:0 DNL was observed for 18:0 percent DNL, and both 14:0 and 18:0 absolute DNL with the severity of the disease (**figures 2.23 & 2.24**). A study conducted by Hellerstein et al reported a non-parallel increase in 18:0 DNL compared to 16:0 DNL in the VLDL (82). These findings were consistent with our data wherein we also observed differences in the percent DNL with the severity when measured in 16:0 of the TRL-TAG but did not observe any changes in 18:0 DNL (**figure 2.23i**). Data from the present study provides evidence that DNL in 14:0 and 18:0 will mirror that in 16:0 when measured in liver-TAG. Further, the total contribution of 14:0 and 18:0 in total absolute DNL was minimal and therefore, the data supports the notion that the

use of 16:0 to measure total DNL is acceptable.

Insulin resistance, FA composition, and DNL indices

Insulin resistance and DNL interplay in NAFLD: Using hyperinsulinemic-euglycemic clamps to measure both hepatic (inverse product of plasma insulin and endogenous glucose appearance rate, Ra) and total body insulin resistance (measured via glucose disposal rate, Rd), Smith et al found that the greater the insulin resistance, the higher the DNL in NAFLD patients (diagnosed via MRI scan) (15). Schwarz et al reported higher DNL in individuals with hyperinsulinemia (11). At the molecular level, insulin promotes DNL through the IRS/PI3K/Akt pathway (86) which increases SREBP1 and FASN expression (87, 88). On the other hand, a DNL precursor, glucose, increased through GLUT2-mediated uptake (89) or increased gluconeogenesis (90, 91) can provide a substrate for the DNL pathway. In the present study, we estimated insulin sensitivity using established calculators (HOMA-IR, QUICKI, and AdipoIR) and found that insulin sensitivity was reduced with NAFLD progression (**figures 2.27a-c**). Moreover, we also observed a negative relationship between peripheral insulin sensitivity and DNL (measured in both liver-TAG and TRL-TAG, **figures 2.28a-b**) similar to the relationship between hepatic insulin sensitivity and DNL observed by Smith et al (15). Additionally, we reported a negative relationship between insulin sensitivity and key DNL enzymes (SREBP1 and FASN, **figures 2.28c-d**). Combined, these data suggest that the relationship observed between insulin resistance and DNL with NAFLD progression was

likely due to changes observed at the molecular level.

DNL was related to MUFA concentrations: Next, individual FAs were quantitated to identify any potential changes in the lipid composition with the severity of the disease. Significant increases observed in the absolute concentrations of individual FAs (14:0, 16:0, 16:1n7, 18:1n7, 18:1n9, and 18:2n6) in liver-TAG and 16:0 and 18:0 in liver-CE with the disease severity is consistent with previous research (92). In general, increased absolute concentrations of SFA, MUFA, and PUFA for both liver-TAG (21) and liver-CE were due to increased total liver-TAG and liver-CE concentrations, respectively, with the disease severity (**table 2.5**). While Puri et al reported increases in omega-6 in NASH patients, we observed significant increases in both omega-3 and omega-6 FAs with the disease severity. Decreases in percent PUFA was perhaps due to increases in percent SFA and MUFA. Previous studies have shown a significant role of low PUFA in NAFLD which led to the development of treatment strategies utilizing PUFA (omega-3 in particular) supplementation for NAFLD patients (93). The ratio of omega-6/omega-3 FAs has been suggested vital for metabolic health and given that, compared to omega-6 PUFA, omega-3 PUFA were more potent promoters of FA oxidation (94-100), which also inhibited malonyl-CoA (98), reduced lipogenesis (95, 97-99), and were found to be lower in NAFLD patients. One study utilized the ratio of n-6/n-3 PUFA as an indicator of FA oxidation and lipogenesis (101), i.e., the higher the ratio, the lower the FA oxidation and greater the lipogenesis. We observed a higher ratio of omega-6/omega-3 FA in liver-

TAG fraction with the severity of the disease (**table 2.5**). We also observed a positive correlation between the liver-TAG omega-6/omega-3 ratio and the NAFLD activity score (**figure 2.30a**). A study conducted by Roumans et al quantitated hepatic FA composition using ¹H-MRS and found a positive relationship between percent SFA and percent DNL measured in the VLDL-TAG. They also found a negative relationship between percent MUFA and percent DNL (102). Although the positive relationship noted with percent SFA was expected based on the fact that the primary product of DNL was 16:0 (SFA) (9, 10, 42, 80-82), a negative relationship reported between DNL and percent MUFA was not consistent with the present study. We found a positive relationship between liver-TAG percent DNL and percent MUFA (**figure 2.30b**) which was also observed previously (58, 103). Both FASN and SCD are increased by insulin. A study conducted by Knebel et al measured DNL indices and MUFA percent in C57Bl6 mice and reported increases in both DNL (measured indirectly) and MUFA. In the study conducted by Lee et al, individuals with high liver fat exhibited a higher mole percent of 16:1n7 in the VLDL and both 16:1n7 and 18:1n7 (MUFA) in NEFA, compared to individuals with low liver fat (58). In the present study, MUFA outcomes were also supported by the gene and protein expression of both FASN and SCD activity (desaturation enzyme that converts SFA to MUFA i.e., 16:0 to 16:1 and 18:0 to 18:1). Both the FASN and SCD (mRNA and protein) expression were related to each other (**figure 2.30c-d**) (104) suggesting FA synthesized via DNL were desaturated to MUFA, and as a result, a stronger relationship was observed between percent DNL and percent MUFA

rather than percent SFA (**figure 2.30b**).

DNL indices may predict the disease severity but do not predict isotopic DNL:

With regard to the indirect measurement of DNL in liver-TAG, only the score obtained through the LD method increased significantly with the severity of the disease (**table 2.6**) and predicted NAFLD activity score ($r=0.660$, $P<0.001$, **table 2.7**). In the liver-CE fraction, the scores from both SCD indices (^{16 & 18}) and EI increased significantly with the disease progression and predicted the disease severity. Importantly, when these indices were compared to isotopic percent DNL, no relationships were observed between DNL indices and isotopic percent DNL suggesting these calculators may not be ideal and cannot replace the gold-standard method for measuring DNL (isotopically). Similar findings were also reported by Rosqvist et al who measured percent DNL isotopically and compared isotopic DNL with LD and SCD₍₁₆₎ index in the VLDL (105). When Lee et al (58) compared individuals with low liver fat vs high liver fat, they found higher LD (reported as DNL index) and SCD₍₁₆₎ index, however, the magnitude of increase observed in LD was low compared to isotopic DNL and SCD₍₁₆₎ index. Further, when they performed correlation analysis, only SCD₍₁₆₎ was associated with isotopic DNL. We observed no relationship with LD and SCD indices in the liver-TAG or TRL-TAG percent DNL. These discrepancies observed between the present study and the previous studies (58, 105) could be due to the fact that the subjects in the present study had higher BMI (around 45kg/m² vs 28-35 kg/m² in other studies), and higher plasma NEFA concentrations (0.90 mmol/L vs 0.50-

0.65 mmol/L) due to negative energy balance (weight-stable bariatric patients' NEFA was 0.60-0.70 mmol/L) (50, 106, 107). Given the higher NEFA concentrations, FA from adipose tissue may have dominated the total liver pool and diluted the presence of DNL-FAs. Combined, these data suggest that the use of DNL indices may not predict percent DNL accurately in individuals undergoing weight loss. Interestingly, a relationship observed between isotopic absolute DNL and DNL indices, LD in liver-TAG, and SCD₍₁₆₎, SCD₍₁₈₎, and EI in liver-CE suggests these indicators may be used to predict absolute DNL but not percent DNL. However, more studies are needed to confirm these findings.

SUMMARY

In summary, liver lipids (both TAG and CE) increased with the severity of the disease. These concepts are depicted in **figure 2.32**. Data from the past studies suggest that elevated liver fat may be due to a combination of 1) constant NEFA flux to the liver, 2) failure to secrete FA out through the TRL particle, and 3) decreased FA oxidation. Moreover, Rd (rate of disposal of glucose) may have resulted in increased utilization of glucose in the DNL pathway resulting in higher DNL with the severity. Although DNL-FAs contribute a small proportion to the liver lipids storage, a direct effect of DNL-FAs was observed on increased liver lipids, NAFLD characteristics, liver enzymes, and decreased FA oxidation. These data suggest that DNL may not necessarily contribute to NAFLD severity through increased lipid storage but rather by altering mechanisms that may induce hepatic pathogenesis in the presence of insulin resistance. Future

research should focus on investigating the precise role of DNL-FAs on individual mechanisms that leads to the development and progression of NAFLD.

LIMITATIONS AND FUTURE RECOMMENDATIONS

This project had a number of limitations. First, the study was conducted in morbidly obese patients undergoing bariatric surgery. Patients were required to lose weight before the procedure and the pre-surgery activities may have altered metabolic pathways in these patients to an extent that the data may not be comparable to other populations. Future research should consider obtaining liver biopsies from weight-stable patients with no more than class II obesity undergoing abdominal surgeries. Second, given that the pre-surgery and post-surgery data for biochemical measurements were collected from the patients' medical records, these data were from different time points for each patient. Third, the DNL measured in 14:0 and 18:0 was highly variable, this was because the ions for all three FAs, 14:0, 16:0, and 18:0, were collected together in a single run. The targeted peak area of 200,000 was achieved for 16:0 but resulted in lower peak areas of 14:0 and 18:0 due to the fact that the 16:0 proportion was higher in any given sample. This may have resulted in a larger variability in 14:0 and 18:0 data. Future research should consider collecting ions for each FA using a separate protocol and targeting a peak area of 200,000 for individual FAs. Fourth, this study included a limited sample size. There were only nine patients in the severe group, although this number was sufficient to achieve the significant differences in the percent DNL, other outcomes in this study (mRNA and protein

expression of enzymes) may not have had a high enough sample size to detect differences. In an effort to recruit nine patients in the severe group, the number of patients recruited in the other two groups was doubled. This was because even though morbid obesity increases the risk of NAFLD/NASH, the presence of a severe form of the disease is rare in these patients. Lastly, because the human biopsies were taken at a single time point, the identification of a precise mechanism through which DNL contributes to the NAFLD development and progression was not possible. However, based on the relationships observed between variables measured in the present study and past literature, potential mechanisms have been elucidated. Future studies should focus on understanding the precise mechanism through which DNL-FAs promote the development and progression of NAFLD to more severe stages.

SIGNIFICANCE OF THE STUDY

The data presented in this study showed for the first time that DNL significantly contributes to NAFLD progression. Because the percent DNL was variable with the severity, i.e., higher in the moderate group and lower in the severe group (particularly in patients with fibrosis) but absolute DNL was the same, DNL inhibitors may promote NAFLD resolution in individuals with any form of the disease. Further, a quarter of the population in the US and other parts of the world are thought to have NAFLD and around 13-25 percent of NASH patients are likely to have fibrosis (108). Therefore, inhibiting DNL may still be of greater significance as a strategy for the treatment of these NAFLD stages. Results from

this study can also be utilized by the pharmaceutical industries and clinical researchers to select the appropriate study population with certain NAFLD characteristics when studying DNL inhibitors. Clinicians can use this information in the future to make an educated decision on the use of DNL inhibitors for the treatment of NAFLD based on the patient's disease severity. Lastly, these results will also serve as preliminary information for researchers investigating the precise role of DNL in NAFLD progression.

Table 2.1: Primer sequences for SYBR Green quantitative real-time PCR

Primer	Sequence 5' ~ 3'	Name/synonym
<i>ABCG8</i>	F: GCA GAT TTC CAA CGA CTT C R: CAT TTG GAG ATG ACA TCC AG	ATP-binding cassette, sub-family-G (WHITE), member-8
<i>ACC1</i>	F: ACA TTA AGA TGG CAG ATC R: CTT GTA CTG GGA TCT TT	Acetyl-coenzyme-A carboxylase-1
<i>ACAT2</i>	F: TCA ATG AAG CCT TTG CAG R: CAA TAT TGA CCT TCT CTG GG	Acetyl-coenzyme-A acetyltransferase-2
<i>APOB</i>	F: CTT ACA TCC TGA ACA TCA AGA G R: AGT TTC CAT ACA CGG TAT CC	Apolipoprotein-B
<i>CD36</i>	F: AGC TTT CCA ATG ATT AGA CG R: GTT TCT ACA AGC TCT GGT TC	Fatty acid translocase
<i>CPT1A</i>	F: TGG ATC TGC TGT ATA TCC R: AAT TGG TTT GAT TTC CTC	Carnitine palmitoyltransferase-1A
<i>DGAT1</i>	F: ATC TTC TTC TAC TGG CTC TTC R: CAG AAG TAG GTG ACA GAC TC	Diacylglycerol o-acyltransferase-1
<i>DGAT2</i>	F: GAG ACT ACT TTC CCA TCC AG R: GAA CTT CTT GCT CAC TTC TG	Diacylglycerol o-acyltransferase-2
<i>FABP1</i>	F: GAA GGT GAC AAT AAA CTG GTG R: GTA TTG GTG ATT ATG TCG CC	Fatty acid binding protein-1
<i>FASN</i>	F: CAA TAC AGA TGG CTT CAA R: GAT GTA TTC AAA TGA CTC	Fatty acid synthase
<i>GAPDH</i>	F: AAC AGC CTC AAG ATC AGC AA R: CAG TCT GGG TGG CAG TGA T	Glyceraldehyde 3-phosphate dehydrogenase
<i>HMGCR</i>	F: ACT TCG TGT TCA TGA CTT TC R: GAC ATA ATC ATC TTG ACC CTC	3-Hydroxy-3-methylglutaryl-coenzyme-A reductase
<i>IL6</i>	F: GCA GAA AAA GGC AAA GAA TC R: CTA CAT TTG CCG AAG AGC	Interleukin-6
<i>INSIG1</i>	F: ACC CCA CAA ATT TAA GAG AG R: TTC TGG AAC GAT CAA ATG TC	Insulin induced gene-1

PRIMER	Sequence 5' ~ 3'	Name/synonym
<i>LDL-R</i>	F: GAG GAC AAA GTA TTT TGG ACA G R: GTA GGT TTT CAG CCA ACA AG	Low-density lipoprotein receptor
<i>MLXIPL</i>	F: GAC CTC AAT TGC TTT TTG TC R: ATC TGA GAT GTC CAT GAA GTC	MLX interacting protein-like/carbohydrate-responsive element-binding protein (ChREBP)
<i>NCEH1</i>	F: CAG TTT ACT CAA GAT GCC AG R: CCT GCA CAA AGT CAT AGT TG	Neutral cholesterol ester hydrolase
<i>PPARA</i>	F: CCT AAA AAG CCT AAG GAA ACC R: GAT CTC CAC AGC AAA TGA TAG	Peroxisome proliferator activated receptor alpha
<i>PPARGC1A</i>	F: GCA GAC CTA GAT TCA AAC TC R: CAT CCC TCT GTC ATC CTC	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
<i>SCD</i>	F: CAG AGG AGG TAC TAC AAA CC R: ATA AGG ACG ATA TCC GAA GAG	Stearoyl-coenzyme A desaturase
<i>SLC27A5</i>	F: CGG TAC TTG TGT AAC ATT CC R: GAC TTC CCA GAT CCG AAT AG	Solute carrier family 27 members-5/ fatty acid transport protein-5 (FATP5)
<i>SOAT2</i>	F: AGA AAG TTT TCA TCA TCC GC R: CTC ATC AAT GAA GTC GAT GG	Sterol o-acyltransferase 2/ acyl-coenzyme A: cholesterol acyltransferase-2 (ACAT2)
<i>SREBF1</i>	F: AAT CTG GGT TTT GTG TCT TC R: AAA AGT TGT GTA CCT TGT GG	Sterol regulatory element binding transcription factor-1
<i>SREBF2</i>	F: CAG CAG GTC AAT CAT AAA CTG R: GGA CAT TCT GAT TAA AGT CCT C	Sterol regulatory element binding transcription factor-2
<i>TNF</i>	F: AGG CAG TCA GAT CAT CTT C R: TTA TCT CTC AGC TCC ACG	Tumor necrosis factor

Table 2.2: Subject characteristics

Subject characteristics	Mild (n=20) (NAS: 0-2)	Moderate (n=20) (NAS: 3-4)	Severe (n=9) (NAS: ≥5)	ANOVA
Age (years)	47.3 ± 11.6	49.4 ± 14.4	53.1 ± 8.1	0.450
BMI (kg/m ²)	46.1 ± 9.9	45.5 ± 6.6	49.5 ± 9.1	0.500
Height (m)	1.69 ± 0.07	1.69 ± 0.08	1.69 ± 0.07	0.898
Body weight (kg)				
Pre-surgery	136.7 ± 30.9	133.4 ± 22.9	145.3 ± 22.8	0.540
Surgery day	131.7 ± 31.2	128.2 ± 21.9	139.6 ± 21.8	0.557
Difference	-5.0 ± 4.4	-5.2 ± 2.6	-5.7 ± 2.8	0.884
Male/Female (n/n)	3/17	5/15	2/7	
Ethnicity (%)				
Whites	90	90	78	
Blacks	10	5	11	
Others	0	5	11	
Hispanic/Latino (%)	0	0	0	
Metabolic syndrome characteristics (n) [§]	2.8 ± 1.2	3.2 ± 1.0	3.1 ± 1.1	0.696
Present (%) ^a	50	70	67	0.402
Hypertension (%) ^a	35	65	56	0.158
Hyperglycemia (%) ^a	35	40	44	0.880
Hypertriglyceridemia (%) ^a	30	15	33	0.431
Low HDLc (%) ^a	80	100	78	0.093
High waist circumference (%) ^a	100	100	100	-
Diabetics (%)	25	40	45	0.500
Hyperlipidemia (%)	15	35	44	0.199
Time of biopsy collected (hh:mm, AM)	9:53 ± 2:34	11:04 ± 2:54	10:02 ± 2:19	-
Fibrosis				
F0 (%) ^b	95	80	22	
F1-F2 (%) ^b	5	10	67	
F3-F4 (%) ^b	0	10	11	

Legend: Data are reported in mean \pm SD. n=49. * $P \leq 0.05$ compared to the mild group, $^\dagger P \leq 0.05$ compared to the moderate group. § The statistical analysis performed and reported here for the metabolic syndrome characteristics are Pearson Chi-square P -value.

^a An individual with \geq three characteristics of metabolic syndrome was considered to have metabolic syndrome; systolic blood pressure ≥ 130 mmHg or diastolic blood pressure ≥ 85 mmHg), plasma glucose >100 mg/dL, triglycerides >150 mg/dL, HDLc <50 mg/dL for women or <40 mg/dL for men, waist circumference >35 inches for women or 40 inches for men. ^b Fibrosis grading: F0 - no fibrosis, F1-F2, mild to moderate fibrosis, F3-F4 - severe fibrosis to cirrhosis. Abbreviations: BMI - body mass index, HDLc - high-density lipoprotein cholesterol, and NAS - NAFLD activity score.

Table 2.3: Liver-related measurements between the groups

Liver-related measurements	Mild (n=20) (NAS: 0-2)	Moderate (n=20) (NAS: 3-4)	Severe (n=9) (NAS: ≥5)	ANOVA
FibroScan® ^a				
CAP (dB/m) ¹	322 ± 55	317 ± 45	382 ± 21*†	0.036
VCTE (kPa) ²	8.4 ± 5.3	7.3 ± 4.4	15.2 ± 10.4†	0.036
Estimated liver weight (g) ^b	1846 ± 395	1808 ± 279	1947 ± 270	0.580
NAFLD calculators ^c				
FS	-0.68 ± 1.84	-0.52 ± 1.85	0.00 ± 0.96	0.619
FIB-4	0.82 ± 0.47	1.01 ± 0.54	1.25 ± 0.50	0.103
HSI	55.7 ± 10.4	55.3 ± 7.6	58.0 ± 9.7	0.764
LFS	-1.1 ± 1.3	-0.1 ± 1.3	2.6 ± 2.8*†	<0.001
DSI	1.15 ± 1.23	1.34 ± 0.97	2.03 ± 0.53	0.111
DSI risk	70 ± 21	76 ± 17	87 ± 5	0.061
FSI	1.82 ± 2.26	2.14 ± 1.52	2.88 ± 1.59	0.375
BARD	3.2 ± 0.7	3.4 ± 0.5	3.4 ± 0.5	0.300
APRI	0.22 ± 1.17	0.28 ± 0.17	0.44 ± 0.22*	0.010
FAST	0.24 ± 0.25	0.23 ± 0.19	0.59 ± 0.31*†	0.009

Legend: Data are reported in mean ± SD. n=49 except for ¹n=35, ²n=32. * $P \leq 0.05$ compared to the mild group, † $P \leq 0.05$ compared to the moderate group. ^a CAP score represents liver fat (a score of 238-260 dB/m represents 11-33% liver fat, 260-290 dB/m represents 34-66% liver fat, >290 dB/m represents >67% liver fat), VCTE score represents fibrosis score (a score of 2-7 kPa represents F0-F1, 7.5-10 kPa represents F2, 10-14 kPa represents F3, >14 represents F4). ^b Estimated liver weight (grams) = [(218 + BW (kg) * 12.3) + (gender*51)] where male = 1, female = 0. ^c Formula for each calculation was listed in the extended methods section. Abbreviations: CAP - controlled attenuation parameter, VCTE - vibration-controlled transient elastography, FS - NAFLD fibrosis score, FIB-4 - fibrosis index based on the 4-factors, HSI - hepatic steatosis index, LFS - NAFLD liver fat score, DSI - Dallas steatosis index, FSI - Framingham steatosis index, BARD - BMI, AST/ALT ratio, diabetes, APRI - aspartate aminotransferase to platelet ratio index, FAST - FibroScan-AST, AST - aspartate transaminase.

Table 2.4: Analytical and biochemical measurements between the groups

Analytical and biochemical measurements	Mild (n=20) (NAS: 0-2)	Moderate (n=20) (NAS: 3-4)	Severe (n=9) (NAS: ≥5)	ANOVA
Deuterium enrichment (%)	0.51 ± 0.15	0.54 ± 0.16	0.52 ± 0.04	0.820
Plasma glucose (mg/dL)				
Pre-surgery ¹	127 ± 84	140 ± 54	153 ± 83	0.686
Surgery day ^a	113 ± 51	114 ± 44	123 ± 48	0.859
Difference ¹	-12 ± 43	-19 ± 44	-30 ± 41	0.587
Insulin (U/L)	6.9 ± 4.0	8.8 ± 2.6	21.4 ± 17.3*†	<0.001
Platelet (thou/μL)	268 ± 76	367 ± 76	252 ± 66	0.856
Hemoglobin A1c (%)				
Pre-surgery ²	6.5 ± 1.4	7.2 ± 1.3	7.1 ± 1.2	0.439
Surgery day	6.2 ± 1.5	6.3 ± 1.3	7.2 ± 2.2	0.261
Difference ²	0.0 ± 0.7	-0.7 ± 0.6*	0.1 ± 0.8	0.029
AST (U/L)				
Pre-surgery ¹	16.8 ± 9.6	21.0 ± 10.1	34.6 ± 13.8*†	0.001
Surgery day ^a	22.1 ± 11.3	28.5 ± 15.2	43.7 ± 23.3*†	0.006
Difference ¹	4.7 ± 16.6	7.4 ± 10.3	9.1 ± 30.3	0.826
ALT (U/L)				
Pre-surgery ¹	22.6 ± 11.3	29.8 ± 16.3	50.9 ± 18.7*†	<0.001
Surgery day	24.5 ± 14.7	34.4 ± 23.4	57.8 ± 24.8*†	0.001
Difference ³¹	2.8 ± 20.1	3.6 ± 13.5	6.9 ± 33.0	0.829
AST/ALT	0.92 ± 0.28	0.94 ± 0.32	0.76 ± 0.15	0.233
ALP (U/L)				
Pre-surgery ¹	77.8 ± 25.6	81.3 ± 26.3	80.0 ± 13.6	0.916
Surgery day ^a	69.4 ± 21.3	68.8 ± 18.5	73.8 ± 21.4	0.822
Difference ¹	-6.4 ± 30.8	-12.9 ± 21.7	-6.2 ± 15.9	0.708
Triglycerides (mg/dL)				
Pre-surgery ³	189 ± 126	175 ± 87	210 ± 90	0.791
Surgery day ^a	145 ± 66	129 ± 61	165 ± 50	0.347
Difference ³	-42 ± 93	-46 ± 37	-51 ± 84	0.963

NEFA (mmol/L)	0.88 ± 0.23	0.90 ± 0.22	0.95 ± 0.23	0.743
Total cholesterol (mg/dL)				
Pre-surgery ³	176 ± 41	175 ± 33	173 ± 15	0.983
Surgery day ^a	161 ± 34	161 ± 31	159 ± 42	0.989
Difference ⁵	-14 ± 28	-21 ± 29	-13 ± 34	0.732
LDLc (mg/dL)				
Pre-surgery ⁴	92 ± 38	93 ± 36	87 ± 18	0.931
Surgery day	96 ± 29	100 ± 27	99 ± 37	0.902
Difference ⁴	3 ± 25	0 ± 26	13 ± 36	0.572
HDLc (mg/dL)				
Pre-surgery ³	52 ± 18	47 ± 10	44 ± 15	0.474
Surgery day ^a	40 ± 13	38 ± 8	37 ± 10	0.661
Difference ³	-11 ± 12	-9 ± 6	-7 ± 11	0.582

Legend: Data are reported in mean ± SD. n=49 except for ¹n=43, ²n=31, ³n=38, ⁴n=37. * $P \leq 0.05$ compared to the mild group, † $P \leq 0.05$ compared to the moderate group. ^a $P \leq 0.05$ compared to pre-surgery (whole group t-test). Abbreviations: AST - aspartate transaminase, ALT - alanine aminotransferases, ALP, alkaline phosphatase, NEFA - nonesterified fatty acids, LDLc - low-density lipoprotein cholesterol, and HDLc - high-density lipoprotein cholesterol.

Table 2.5: Lipid subclass analysis in the liver and the TRL particle across the disease severity

Lipid class	Mild (n=20) (NAS: 0-2)	Moderate (n=20) (NAS: 3-4)	Severe (n=9) (NAS: ≥5)	ANOVA
Liver-TAG				
SFA	9.1 ± 6.4	51.8 ± 44.0*	110.5 ± 60.1*†	<0.001
Percent	31.7 ± 2.0	33.0 ± 2.6	37.9 ± 2.9*†	<0.001
MUFA	13.2 ± 9.3	75.8 ± 64.5*	133.4 ± 55.0*†	<0.001
Percent	44.6 ± 3.8	48.4 ± 2.4*	47.7 ± 3.1*	0.001
PUFA	6.7 ± 4.6	27.4 ± 23.1*	42.4 ± 22.4*	<0.001
Percent	23.7 ± 3.3	18.6 ± 3.2*	14.4 ± 1.9*†	<0.001
Omega-3 FA	0.3 ± 0.2	1.1 ± 0.9*	1.7 ± 1.1*	<0.001
Percent	1.4 ± 0.6	0.8 ± 0.2*	0.5 ± 0.2*	<0.001
Omega-6 FA	6.3 ± 4.4	26.3 ± 22.2*	40.7 ± 21.3*	<0.001
Percent	22.3 ± 3.0	17.8 ± 3.0*	13.9 ± 1.7*†	<0.001
Omega-6/omega-3 ratio	18.8 ± 5.8	25.3 ± 5.4*	26.8 ± 6.3*	<0.001
EPA+DHA	0.1 ± 0.1	0.1 ± 0.2	0.0 ± 0.0	0.057
Percent	0.5 ± 0.5	0.1 ± 0.2*	0.0 ± 0.0*	<0.001
Essential FA	6.7 ± 4.6	27.4 ± 23.1*	42.4 ± 22.4*	<0.001
Non-essential FA	22.3 ± 15.6	127.6 ± 108.3*	243.9 ± 114.0*†	<0.001
Liver-CE				
SFA	4.1 ± 1.7	6.7 ± 2.7*	8.5 ± 4.4*	<0.001
Percent	23.2 ± 6.7	23.1 ± 7.8	17.6 ± 2.5	0.093
MUFA	4.1 ± 1.4	8.2 ± 4.6*	12.2 ± 4.6*†	<0.001
Percent	23.7 ± 5.0	26.2 ± 4.3	26.6 ± 1.7	0.115
PUFA	9.8 ± 5.8	17.2 ± 13.4	25.6 ± 9.2*	0.001
Percent	53.1 ± 7.6	50.6 ± 8.3	55.8 ± 2.9	0.213
Omega-3 FA	1.4 ± 1.3	1.6 ± 1.7	1.6 ± 0.6	0.973
Percent	7.1 ± 5.0	5.5 ± 4.5	4.0 ± 1.8	0.203
Omega-6 FA	8.5 ± 4.8	15.6 ± 13.1	24.0 ± 9.3*	0.001
Percent	46.0 ± 7.8	45.2 ± 8.3	51.8 ± 2.3	0.083

Lipid class	Mild (n=20) (NAS: 0-2)	Moderate (n=20) (NAS: 3-4)	Severe (n=9) (NAS: ≥5)	ANOVA
Omega-6/omega-3 ratio ¹	8.5 ± 8.4	16.8 ± 21.5	26.2 ± 36.5	0.059
EPA+DHA	0.3 ± 0.2	0.3 ± 0.1	0.3 ± 0.2	0.142
Percent	1.6 ± 1.1	1.1 ± 0.6	0.6 ± 0.2*	0.008
Essential FA	9.8 ± 5.8	17.2 ± 13.4	25.6 ± 9.2*	0.001
Non-essential FA	8.1 ± 2.5	14.9 ± 6.8*	20.7 ± 8.9*	<0.001
Liver-FFA				
SFA	1.2 ± 0.6	1.3 ± 0.5	1.4 ± 0.5	0.794
Percent	52.9 ± 24.6	59.4 ± 20.9	52.0 ± 23.6	0.598
MUFA	0.4 ± 0.5	0.2 ± 0.2	0.3 ± 0.3	0.197
Percent	11.8 ± 9.8	7.3 ± 6.0	9.8 ± 5.8	0.198
PUFA	1.1 ± 0.8	0.8 ± 0.5	1.3 ± 1.0	0.265
Percent	35.3 ± 17.3	33.3 ± 16.5	38.2 ± 18.2	0.774
Omega-3 FA	0.5 ± 0.4	0.4 ± 0.2	0.6 ± 0.5	0.228
Percent	15.5 ± 8.2	14.8 ± 8.1	16.0 ± 8.7	0.927
Omega-6 FA	0.6 ± 0.4	0.5 ± 0.3	0.8 ± 0.6	0.209
Percent	19.9 ± 9.6	18.6 ± 8.7	22.1 ± 10.2	0.641
Omega-6/omega-3 ratio ²	1.5 ± 0.8	1.4 ± 0.4	1.5 ± 0.8	0.785
EPA+DHA	5 ± 7	4 ± 6	7 ± 7	-
Percent	0.3 ± 0.3	0.4 ± 0.6	0.5 ± 0.6	0.549
Essential FA	1.1 ± 0.8	0.8 ± 0.5	1.3 ± 1.0	0.265
Non-essential FA	1.6 ± 0.8	1.5 ± 0.5	1.7 ± 0.4	0.577
TRL-TAG				
SFA (mg/dL)	70 ± 48	68 ± 59	87 ± 35	0.630
Percent	29.4 ± 5.7	31.5 ± 2.1	32.5 ± 2.0	0.105
MUFA (mg/dL)	104 ± 73	97 ± 87	121 ± 52	0.726
Percent	42.8 ± 5.0	43.7 ± 3.6	45.0 ± 2.0	0.386
PUFA (mg/dL)	66 ± 45	48 ± 25	60 ± 27	0.241
Percent	27.8 ± 2.9	24.7 ± 4.4*	22.5 ± 2.4*	0.001
Omega-3 FA (mg/dL)	3 ± 2	3 ± 2	3 ± 1	0.858

Lipid class	Mild (n=20) (NAS: 0-2)	Moderate (n=20) (NAS: 3-4)	Severe (n=9) (NAS: ≥5)	ANOVA
Percent	1.5 ± 0.4	1.4 ± 0.7	1.1 ± 0.3	0.241
Omega-6 FA (mg/dL)	63 ± 43	45 ± 24	58 ± 26	0.231
Percent	26.4 ± 2.9	23.3 ± 4.4*	21.4 ± 2.3*	0.002
Omega-6/omega-3 ratio	20 ± 6	19 ± 8	20 ± 4	0.936
EPA+DHA (mg/dL)	0.5 ± 0.5	0.5 ± 0.5	0.5 ± 0.4	0.454
Percent	0.3 ± 0.3	0.3 ± 0.3	0.2 ± 0.2	0.763
Essential FA (mg/dL)	66 ± 45	48 ± 25	60 ± 27	0.241
Non-essential FA (mg/dL)	174 ± 117	165 ± 145	208 ± 87	0.689

Legend: Data are reported in mean ± SD. n=49 except ¹n=44, ²n=42

Values are in mg/g of tissue (wet weight) for liver and mg/dL for TRL

* $P \leq 0.05$ compared to the mild group, † $P \leq 0.05$ compared to the moderate group

SFA included the sum of 14:0, 16:0, and 18:0 FA

MUFA included the sum of 16:1n7, 18:1n9, and 18:1n7 FA

PUFA included the sum of 18:2n6, 18:3n6, 18:3n3, 20:3n6, 20:4n6, 20:5n3, 22:4n6, 22:5n6, 22:5n3, and 22:6n3 FA

Omega-3 FA included the sum of 18:3n3, 20:5n3, 22:5n3, and 22:6n3 FA

Omega-6 FA included the sum of 18:2n6, 18:3n6, 20:3n6, 20:4n6, 22:4n6, and 22:5n6 FA

Essential FA included the sum of 18:2n6, 18:3n6, 18:3n3, 20:3n6, 20:4n6, 20:5n3, 22:4n6, 22:5n6, 22:5n3, and 22:6n3 FA

non-essential FA included the sum of 14:0, 16:0, 16:1n7, 18:0, 18:1n9, and 18:1n7 FA

Abbreviations: TG - triglycerides, SFA - saturated fatty acids, MUFA - monounsaturated fatty acids, PUFA - polyunsaturated fatty acids, FA - fatty acids, EPA - eicosapentaenoic acid, DHA - docosahexaenoic acid, CE - cholesterol, FFA - free fatty acids.

Table 2.6: Calculated DNL indices across disease severity

Analytical measurements	Mild (n=20) (NAS: 0-2)	Moderate (n=20) (NAS: 3-4)	Severe (n=9) (NAS: ≥5)	ANOVA
DNL indices				
LD (16:0/18:2)				
Liver-TAG	1.43 ± 0.25	1.81 ± 0.42*	2.53 ± 0.44*†	<0.001
Liver-CE	0.78 ± 0.58	0.69 ± 0.60	0.34 ± 0.04	0.133
Liver-FFA ¹	34.87 ± 48.08	26.77 ± 21.06	14.69 ± 11.07	0.474
TRL-TAG ²	15.03 ± 10.30	13.94 ± 6.75	25.06 ± 19.78	0.054
TRL-CE	0.59 ± 0.71	0.51 ± 0.43	0.50 ± 0.42	0.890
SCD ₍₁₆₎ Index (16:1n7/16:0)				
Liver-TAG	0.11 ± 0.03	0.11 ± 0.03	0.13 ± 0.04	0.279
Liver-CE	0.12 ± 0.06	0.17 ± 0.09	0.30 ± 0.13*†	<0.001
Liver-FFA	0.10 ± 0.10	0.05 ± 0.05	0.10 ± 0.16	0.168
TRL-TAG	0.97 ± 0.49	1.01 ± 0.43	0.89 ± 0.50	0.822
TRL-CE ³	0.15 ± 0.05	0.17 ± 0.07	0.27 ± 0.22*	0.038
SCD ₍₁₈₎ Index (18:1n9/18:0)				
Liver-TAG	11.18 ± 2.55	15.95 ± 19.30	9.95 ± 2.05	0.366
Liver-CE	4.10 ± 1.29	5.76 ± 2.26*	7.86 ± 1.31*†	<0.001
Liver-FFA ⁴	0.41 ± 0.88	0.17 ± 0.18	0.53 ± 0.89	0.363
TRL-TAG	0.06 ± 0.02	0.07 ± 0.01	0.08 ± 0.01	0.064
TRL-CE ³	8.27 ± 5.01	7.42 ± 3.98	7.50 ± 5.79	0.847
Elongation Index (18:0/16:0)				
Liver-TAG	0.14 ± 0.03	0.14 ± 0.05	0.14 ± 0.04	0.999
Liver-CE	0.31 ± 0.10	0.23 ± 0.05*	0.19 ± 0.04*	<0.001
Liver-FFA	0.55 ± 0.25	0.48 ± 0.18	0.50 ± 0.17	0.589
TRL-TAG	13.34 ± 5.44	13.21 ± 4.03	11.27 ± 3.93	0.508
TRL-CE ³	0.25 ± 0.20	0.24 ± 0.13	0.35 ± 0.22	0.316

Analytical measurements	Mild (n=20) (NAS: 0-2)	Moderate (n=20) (NAS: 3-4)	Severe (n=9) (NAS: ≥5)	ANOVA
Elongation index (18:1n7/16:1n7)				
Liver-TAG	0.93 ± 0.27	0.93 ± 0.29	0.72 ± 0.20	0.136
Liver-CE	1.81 ± 1.36	1.19 ± 1.82	0.47 ± 0.26	0.077
Liver-FFA	2.58 ± 1.21	2.17 ± 1.65	2.20 ± 1.70	0.731
TRL-TAG	9.03 ± 1.55	7.58 ± 2.40	7.55 ± 4.58	0.175
TRL-CE ³	1.80 ± 1.74	1.42 ± 0.91	1.69 ± 1.11	0.671

Legend: Data are reported in mean ± SD. n=49 except ¹n=41, ²n=48, ³n=47, ⁴n=34 * $P \leq 0.05$ compared to the mild group, † $P \leq 0.05$ compared to the moderate group. Abbreviations: TG - triglycerides, CE - cholesterol, FFA - free fatty acids.

Table 2.7: Correlation analysis of DNL indices with isotopic measurements of DNL

DNL Indices		NAS	Liver-TAG %DNL	Liver-TAG aDNL	Liver-CE %DNL	Liver-CE aDNL	Liver-FFA %DNL	Liver-FFA aDNL	TRL-TAG %DNL	TRL-TAG aDNL	TRL-CE %DNL
LD (16:0/18:2)											
Liver-TAG	R	0.660	-0.057	0.514	0.100	0.257	-0.048	-0.020	-0.004	0.216	0.111
	P	<0.001	0.700	<0.001	0.494	0.075	0.741	0.892	0.976	0.136	0.446
	n	49	49	47	49	49	49	49	49	49	49
Liver-CE	R	-0.372^a	0.135	-0.212	0.201	-0.099	0.337	0.281	0.161	0.177	0.072
	P	0.009	0.356	0.153	0.166	0.497	0.018	0.050	0.269	0.224	0.622
	n	49	49	47	49	49	49	49	49	49	49
Liver-FFA	R	-0.198	-0.022	-0.111	0.044	-0.065	0.028	-0.209	-0.109	-0.066	0.034
	P	0.214	0.889	0.495	0.784	0.687	0.861	0.191	0.498	0.680	0.833
	n	41	41	40	41	41	41	41	41	41	41
TRL-TAG	R	0.148	-0.117	-0.034	0.238	0.157	-0.255	-0.173	-0.111	-0.163	-0.038
	P	0.314	0.430	0.824	0.103	0.285	0.081	0.240	0.454	0.268	0.795
	n	48	48	46	48	48	48	48	48	48	48
TRL-CE	R	-0.044	0.011	-0.006	0.242	0.325	0.120	0.001	0.156	-0.042	0.160
	P	0.766	0.942	0.970	0.095	0.023	0.410	0.993	0.286	0.777	0.271
	n	49	49	47	49	49	49	49	49	49	49
SCD ₍₁₆₎ Index (16:1n7/16:0)											
Liver-TAG	R	0.098	0.125	0.177	0.142	0.038	0.115	-0.082	.283	0.274	0.278
	P	0.503	0.394	0.235	0.330	0.793	0.430	0.575	0.049	0.057	0.054
	n	49	49	47	49	49	49	49	49	49	49
Liver-CE	R	0.480	-0.071	0.331	0.056	0.318	-0.059	-0.041	0.152	0.164	0.123
	P	<0.001	0.630	0.023	0.700	0.026	0.686	0.777	0.298	0.259	0.399
	n	49	49	47	49	49	49	49	49	49	49
Liver-FFA	R	-0.059	-0.180	-0.240	0.222	0.183	-0.043	0.393	0.043	-0.020	<0.001

	<i>P</i>	0.688	0.216	0.104	0.126	0.208	0.768	0.005	0.771	0.890	0.999
	<i>n</i>	49	49	47	49	49	49	49	49	49	49
TRL-TAG	<i>R</i>	0.114	-0.013	0.015	-0.024	0.179	-0.090	0.185	-0.059	-0.058	-0.151
	<i>P</i>	0.435	0.932	0.922	0.871	0.217	0.538	0.203	0.685	0.693	0.300
	<i>n</i>	49	49	47	49	49	49	49	49	49	49
TRL-CE	<i>R</i>	0.324	-0.153	-0.007	-0.079	0.145	-0.084	0.073	0.055	0.159	-0.062
	<i>P</i>	0.026	0.305	0.961	0.596	0.332	0.574	0.628	0.715	0.285	0.679
	<i>n</i>	47	47	46	47	47	47	47	47	47	47
SCD ₍₁₈₎ Index (18:1n9/18:0)											
Liver-TAG	<i>R</i>	0.073	0.168	0.119	0.036	-0.031	0.020	-0.059	0.141	0.138	0.214
	<i>P</i>	0.619	0.249	0.425	0.805	0.835	0.892	0.689	0.334	0.345	0.140
	<i>n</i>	49	49	47	49	49	49	49	49	49	49
Liver-CE	<i>R</i>	0.576	0.040	0.462	0.121	0.608	-0.225	-0.204	0.086	0.096	0.069
	<i>P</i>	<0.001	0.783	0.001	0.406	<0.001	0.120	0.160	0.559	0.514	0.635
	<i>n</i>	49	49	47	49	49	49	49	49	49	49
Liver-FFA	<i>R</i>	-0.027	-0.133	-0.105	0.118	0.101	-0.007	0.441	0.030	-0.128	-0.113
	<i>P</i>	0.856	0.361	0.481	0.419	0.491	0.960	0.002	0.837	0.381	0.438
	<i>n</i>	49	49	47	49	49	49	49	49	49	49
TRL-TAG	<i>R</i>	0.298	0.025	0.136	0.018	-0.095	0.151	-0.004	0.216	0.208	0.309
	<i>P</i>	0.038	0.864	0.361	0.902	0.514	0.299	0.978	0.136	0.151	0.031
	<i>n</i>	49	49	47	49	49	49	49	49	49	49
TRL-CE	<i>R</i>	-0.008	-0.052	-0.024	-0.201	-0.254	-0.003	0.194	0.119	0.430	0.077
	<i>P</i>	0.955	0.729	0.875	0.175	0.085	0.982	0.191	0.424	0.003	0.606
	<i>n</i>	47	47	46	47	47	47	47	47	47	47
Elongation index (18:0/16:0)											
Liver-TAG	<i>R</i>	0.015	-0.120	-0.198	-0.131	-0.033	-0.210	0.066	-0.122	-0.076	-0.210
	<i>P</i>	0.920	0.412	0.181	0.369	0.825	0.148	0.650	0.404	0.603	0.147
	<i>n</i>	49	49	47	49	49	49	49	49	49	49
Liver-CE	<i>R</i>	-0.489	-0.270	-0.404	-0.420	-0.553	0.030	0.008	-0.219	-0.182	-0.192

	<i>P</i>	<0.001	0.060	0.005	0.003	<0.001	0.837	0.959	0.130	0.211	0.186
	<i>n</i>	49	49	47	49	49	49	49	49	49	49
Liver-FFA	<i>R</i>	-0.101	-0.030	-0.142	-0.203	-0.126	0.002	0.005	0.038	-0.092	-0.108
	<i>P</i>	0.489	0.841	0.341	0.161	0.388	0.988	0.975	0.797	0.531	0.462
	<i>n</i>	49	49	47	49	49	49	49	49	49	49
TRL-TAG	<i>R</i>	0.026	0.029	-0.021	-0.080	0.022	-0.080	0.148	-0.120	-0.074	-0.206
	<i>P</i>	0.857	0.843	0.890	0.585	0.880	0.586	0.309	0.413	0.614	0.157
	<i>n</i>	49	49	47	49	49	49	49	49	49	49
TRL-CE	<i>R</i>	0.146	-0.187	-0.136	-0.008	0.177	-0.207	-0.260	-0.289	-0.349	-0.194
	<i>P</i>	0.327	0.208	0.366	0.956	0.234	0.163	0.078	0.049	0.016	0.192
	<i>n</i>	47	47	46	47	47	47	47	47	47	47
Elongation Index (18:1n7/16:1n7)											
Liver-TAG	<i>R</i>	-0.086	-0.016	-0.306	-0.102	-0.153	-0.035	0.086	-0.110	-0.152	-0.100
	<i>P</i>	0.559	0.913	0.037	0.487	0.295	0.814	0.558	0.451	0.296	0.496
	<i>n</i>	49	49	47	49	49	49	49	49	49	49
Liver-CE	<i>R</i>	-0.290	-0.080	-0.295	-0.113	-0.148	0.080	0.151	-0.068	-0.086	-0.146
	<i>P</i>	0.043	0.584	0.044	0.438	0.310	0.586	0.302	0.644	0.556	0.315
	<i>n</i>	49	49	47	49	49	49	49	49	49	49
Liver-FFA	<i>R</i>	-0.022	-0.003	-0.191	-0.164	-0.136	0.058	0.191	0.017	0.186	0.103
	<i>P</i>	0.901	0.988	0.288	0.353	0.444	0.744	0.279	0.926	0.291	0.561
	<i>n</i>	34	34	33	34	34	34	34	34	34	34
TRL-TAG	<i>R</i>	-0.307	-0.138	-0.239	-0.231	-0.240	-0.006	-0.037	-0.209	-0.302	-0.233
	<i>P</i>	0.032	0.346	0.106	0.111	0.096	0.968	0.803	0.149	0.035	0.107
	<i>n</i>	49	49	47	49	49	49	49	49	49	49
TRL-CE	<i>R</i>	-0.098	-0.246	-0.220	0.059	0.052	-0.019	0.124	-0.294	-0.395	-0.242
	<i>P</i>	0.514	0.095	0.142	0.694	0.728	0.900	0.405	0.045	0.006	0.101
	<i>n</i>	47	47	46	47	47	47	47	47	47	47

Legend: Data presented are *P*-values and *R*. ^a *r*=-0.285, *P*=0.047 (when outlier was included).

Figure 2.1: Consort flow

CONSORT

TRANSPARENT REPORTING of TRIALS

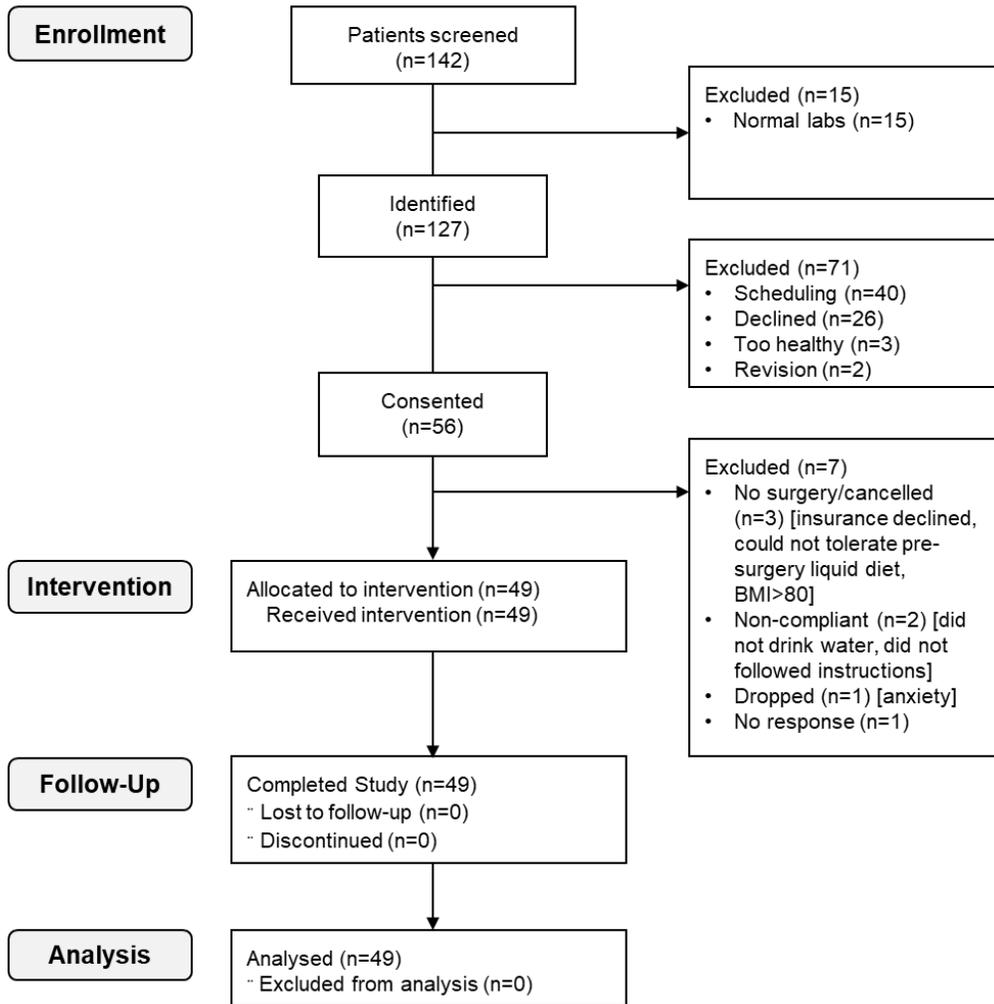


Figure 2.2: Study design

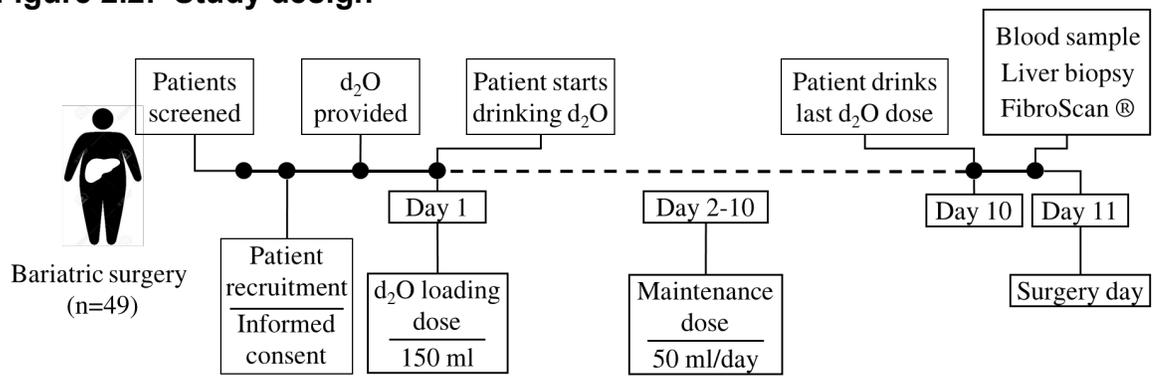
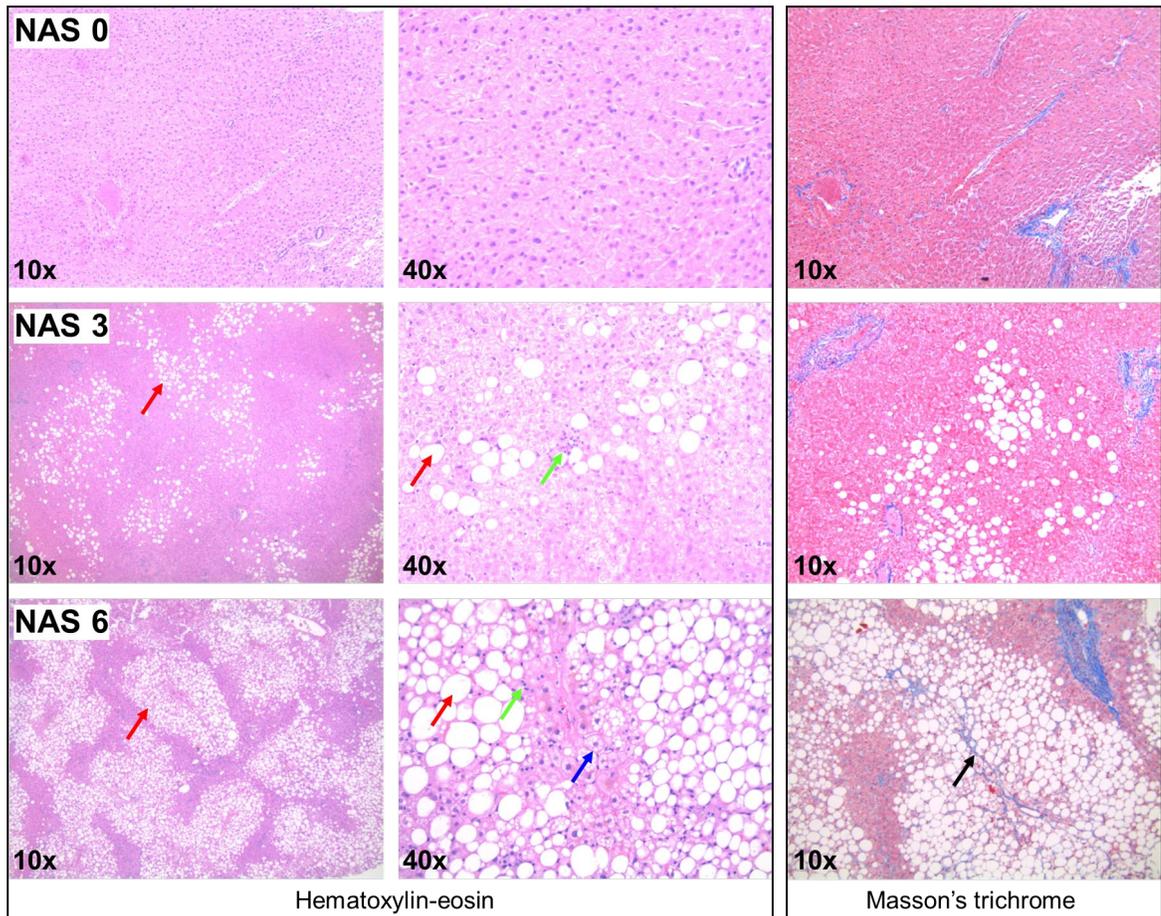


Figure 2.3: NAFLD activity score system and histology



Legend: Histologic examples of the NAFLD activity score (NAS) system. Left two columns: Hematoxylin-eosin staining used to identify steatosis, inflammation, and ballooning. Middle column: a magnified version of the micrographs on the left. Right column: Masson's trichrome staining used to identify fibrosis.

In the figures, one can appreciate:

- Steatosis: signified by white droplets (indicated with red arrows)
- Inflammatory cells: dark dots within a cell (green arrows)
- Cell death (ballooning): enlarged cells (blue arrow)
- Fibrosis: visible with blue staining (black arrow)

The top row shows data from a patient with a NAS of zero (healthy).

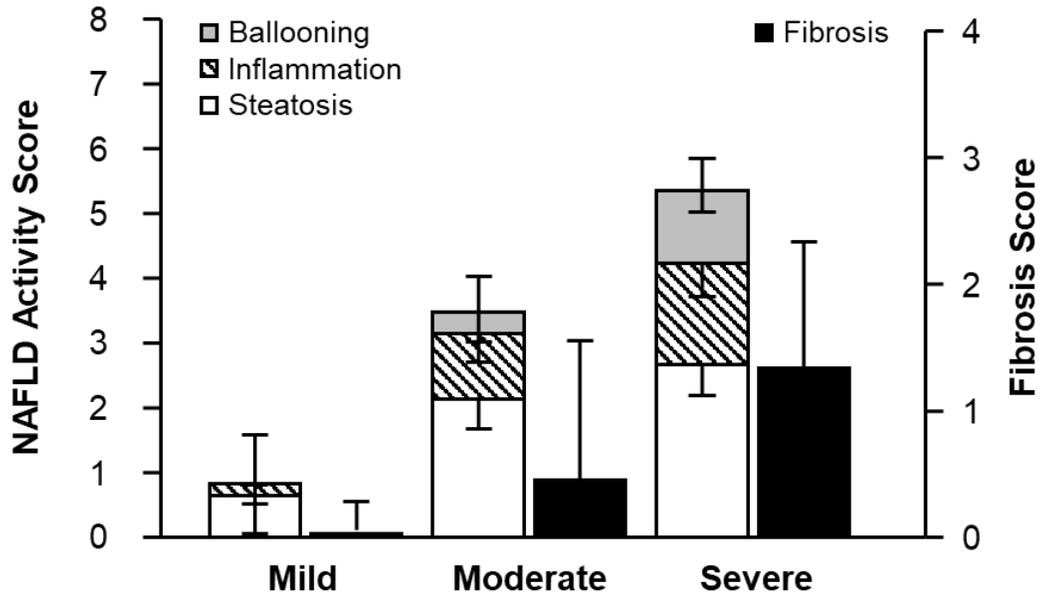
Second row: patient with a NAS of 3.

- Steatosis and inflammation are moderate but no ballooning or fibrosis present.

Third row: patient with a NAS of 6.

- Steatosis and inflammation are severe.
- Ballooning and fibrosis are mild.

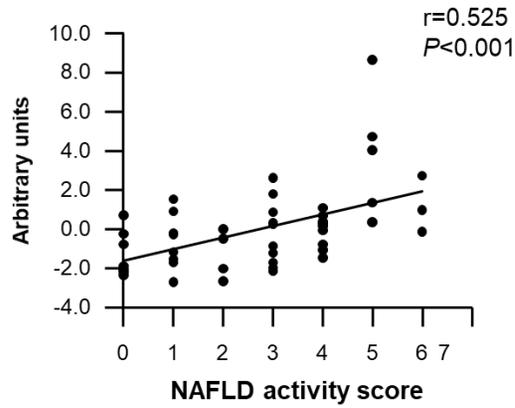
Figure 2.4: NALFD activity score and fibrosis score in each group



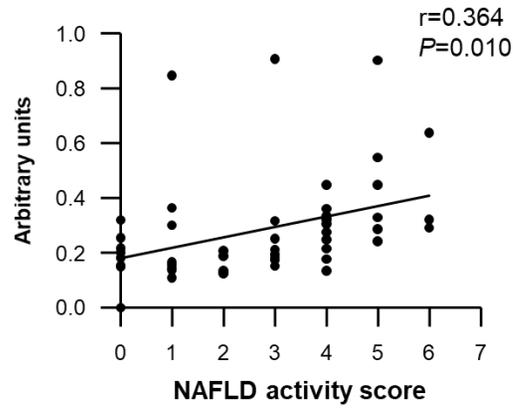
Legend: Data are presented in mean±SD. Clustered bars on the left side represent the combination of steatosis, inflammation, and ballooning scores. Individual bars next to clustered bars represent the fibrosis score (secondary axis). With an increase in severity, each characteristic's score increased significantly.

Figure 2.5: NAFLD calculators and histological scoring

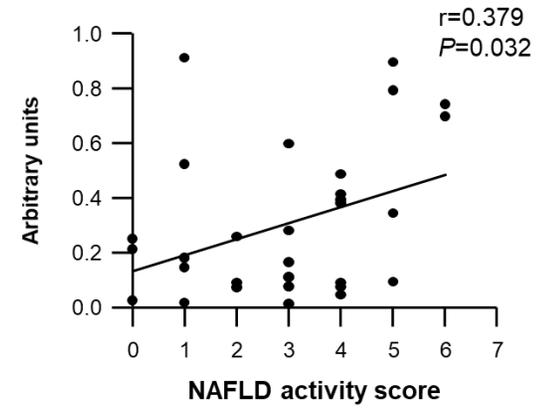
a. LFS



b. APRI score

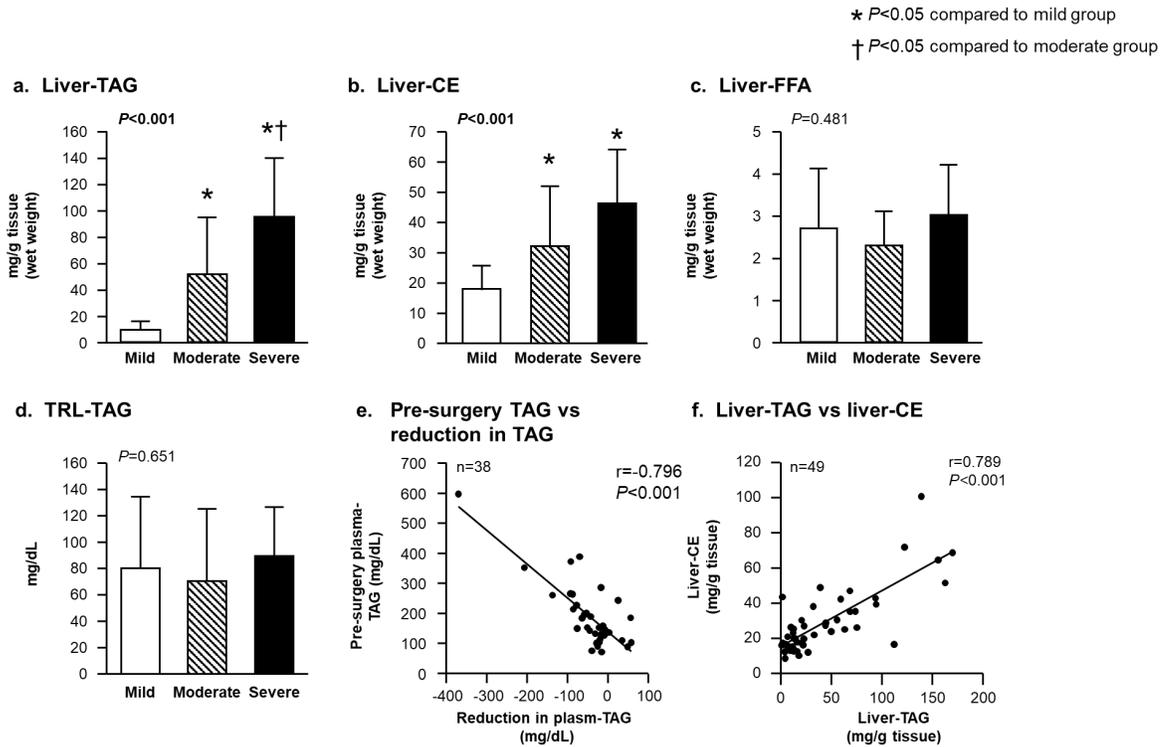


c. FAST score



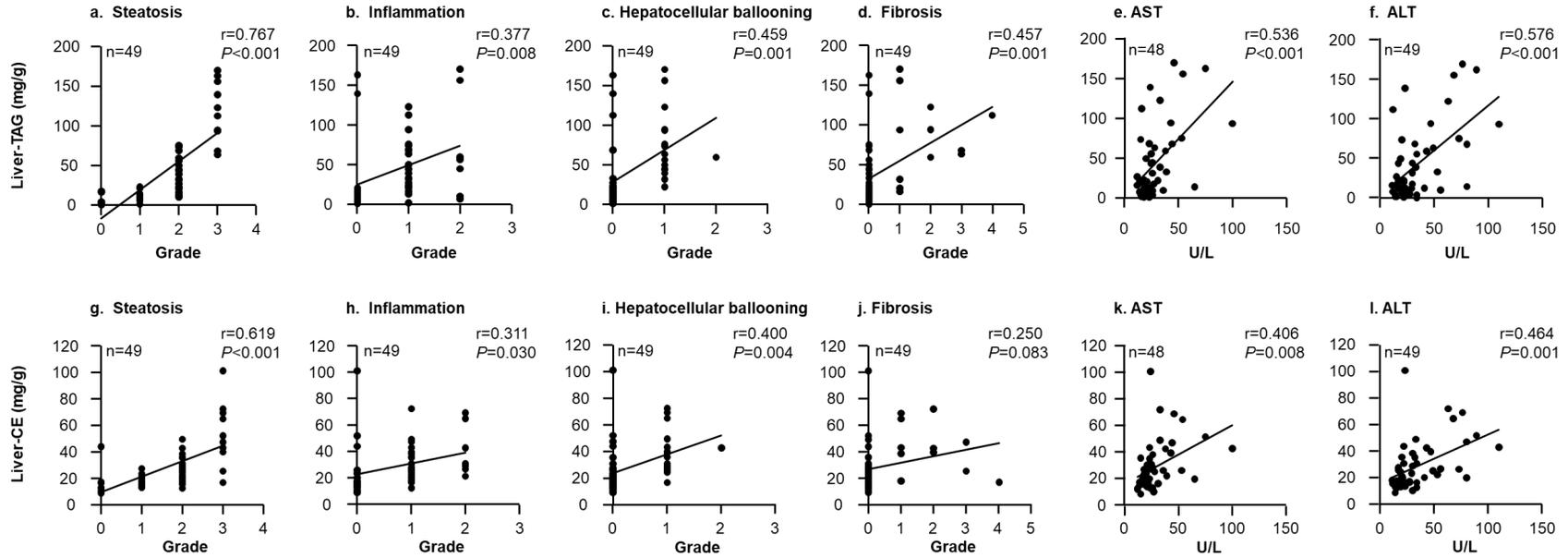
Legend: n=49. LFS, APRI, and FAST score calculations are presented in the methods section. A bivariate Pearson correlation analysis with two-tail significance was performed to test the correlation between NAFLD activity score, derived from histological evaluation, with LFS, APRI, and FAST scores.

Figure 2.6: Lipid content of the liver and the TRL particle



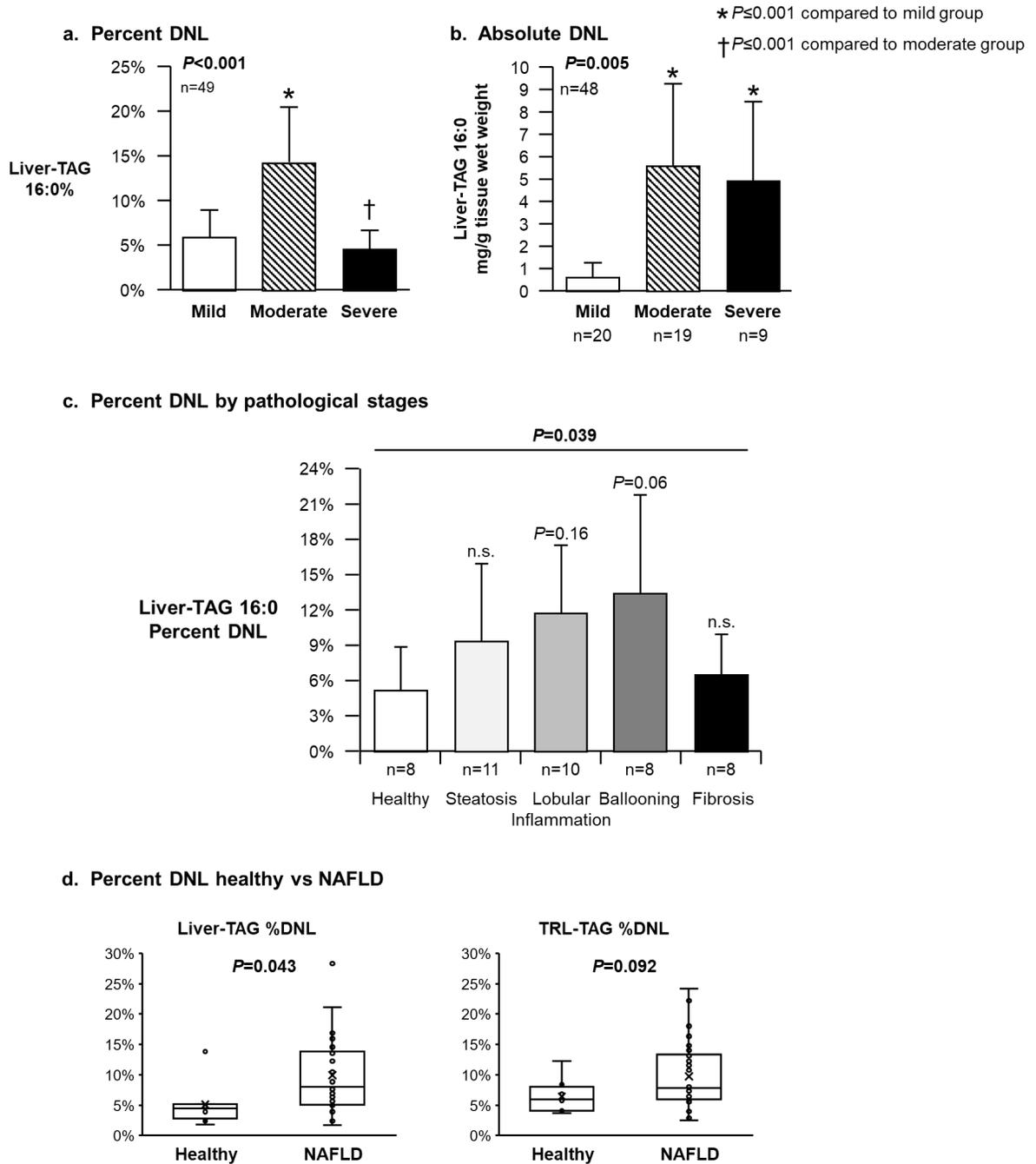
Legend: Figures a-d: $n = 49$. Data are reported in mean \pm SD. One-way ANOVA was performed between the groups and the P -value was presented above each bar-graph. If significant, the Tukey post-hoc analysis was performed to test the significance of each group. * $P < 0.05$ compared to the mild group. † $P < 0.05$ compared to the moderate group. Figures e-f: n = different for each panel. A bivariate Pearson correlation analysis with two-tail significance was performed to test the correlation between pre-surgery plasma-TAG and reduction in plasma-TAG; and between liver-CE and liver-TAG.

Figure 2.7: Liver lipids concentrations across NAFLD stages



Legend: n=different for each panel. A bivariate Pearson correlation analysis with two-tail significance was performed to test the correlation between liver-TAG and liver-CE with steatosis, inflammation, hepatocellular ballooning, fibrosis, AST, and ALT.

Figure 2.8: De novo lipogenesis in liver-TAG

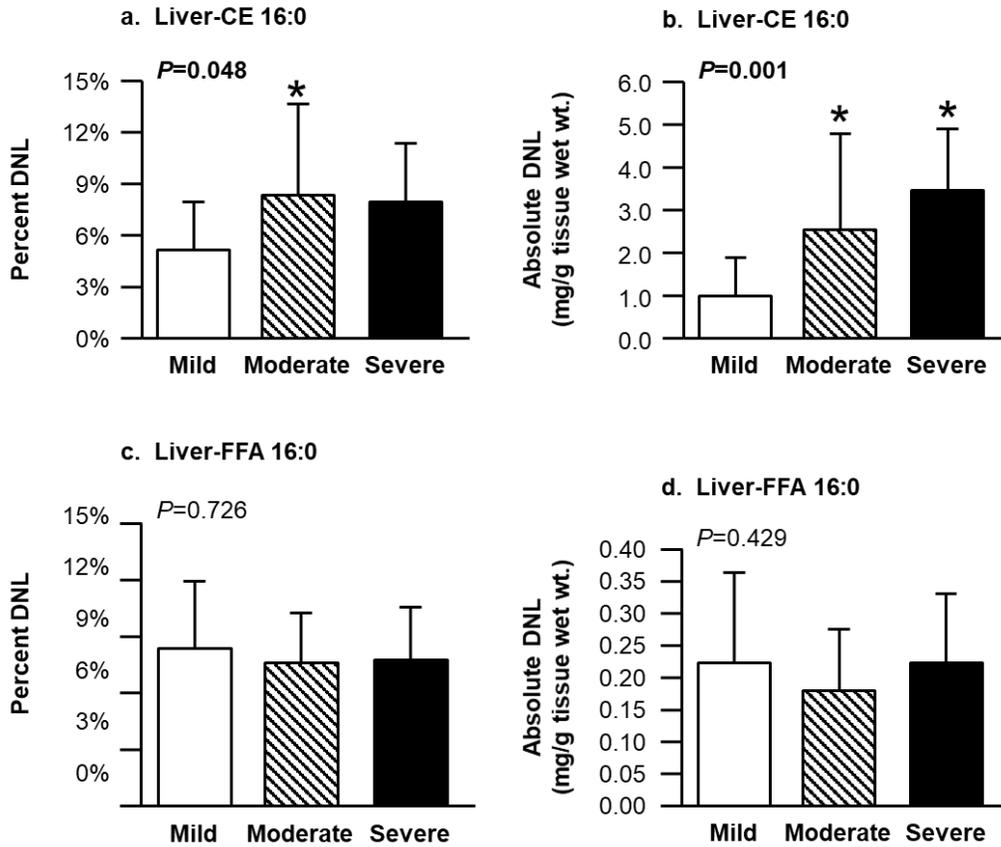


Legend a-b: n=different for each panel. Data are reported in mean±SD. Absolute DNL was calculated by multiplying percent DNL with total liver-TAG concentrations. One-way ANOVA was performed between the groups and the P -value was presented above each bar-graph. If significant, the Tukey post-hoc analysis was performed to test the significance of each group. * $P \leq 0.001$ compared to the mild group. † $P \leq 0.001$ compared to the moderate group. For

the absolute DNL moderate group, one patient's DNL was removed as an outlier (n=19). **c)** n=45. Data are reported in mean±SD. One-way ANOVA was performed between the groups and the *P*-value was presented above each bar-graph. If significant, the Tukey post-hoc analysis was performed to test the significance of each group. Four subjects' data were not included because they did not follow the typical progression of NAFLD (steatosis, inflammation, ballooning, and fibrosis). **d)** n=49. Data are reported in mean±SD. One-way ANOVA was performed between the groups and the *P*-value was presented above each bar-graph.

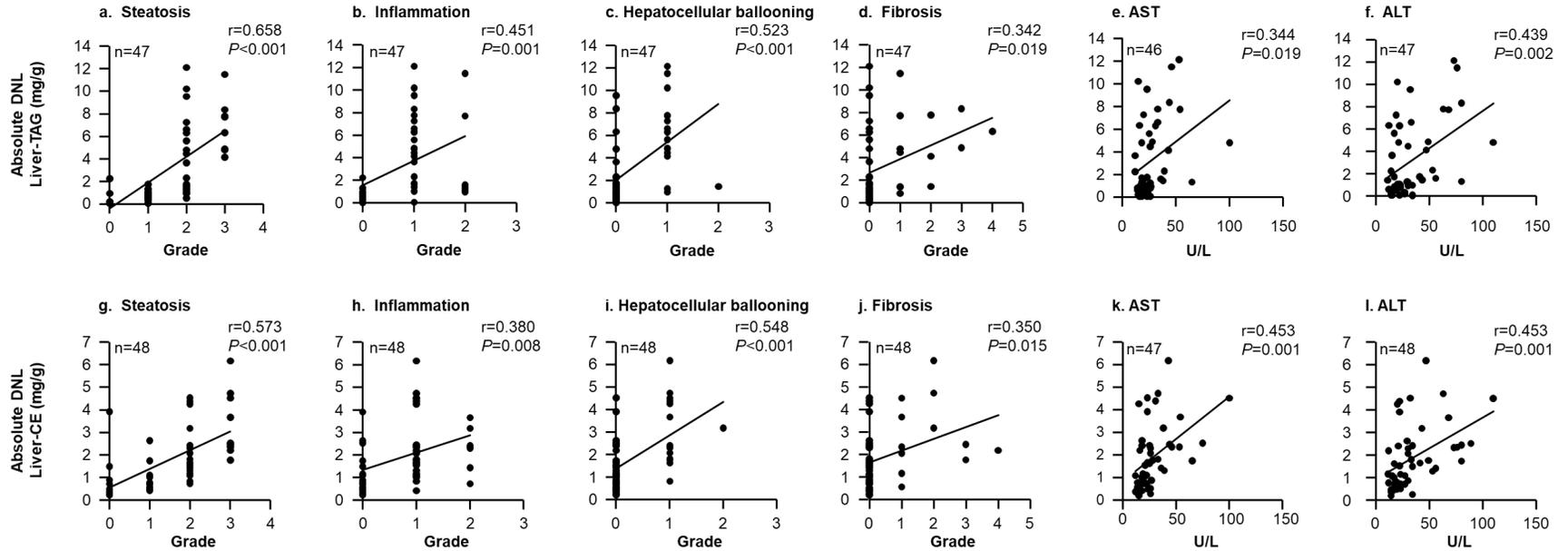
Figure 2.9: De novo lipogenesis in the other liver lipids

* $P < 0.05$ compared to mild group



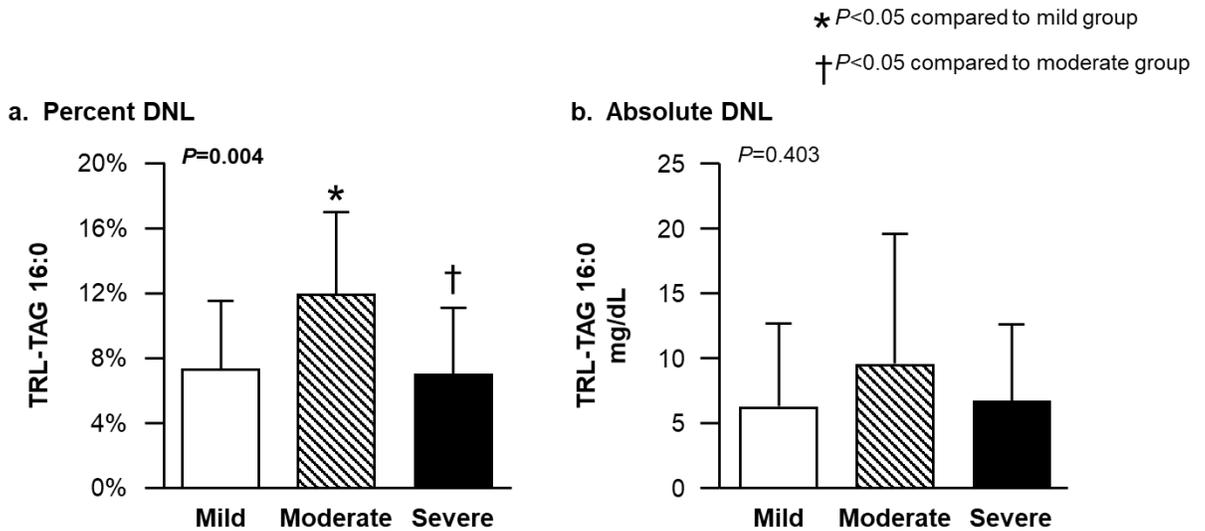
Legend: n=49. Data are reported in mean \pm SD. Absolute DNL was calculated by multiplying percent DNL with total liver-CE or FFA concentrations, respectively. One-way ANOVA was performed between the groups and the P -value was presented above each bar-graph. If significant, the Tukey post-hoc analysis was performed to test the significance of each group. * $P < 0.05$ compared to the mild group. For panel a. $P = 0.221$ for the difference between the mild and severe groups.

Figure 2.10: DNL in NAFLD stages



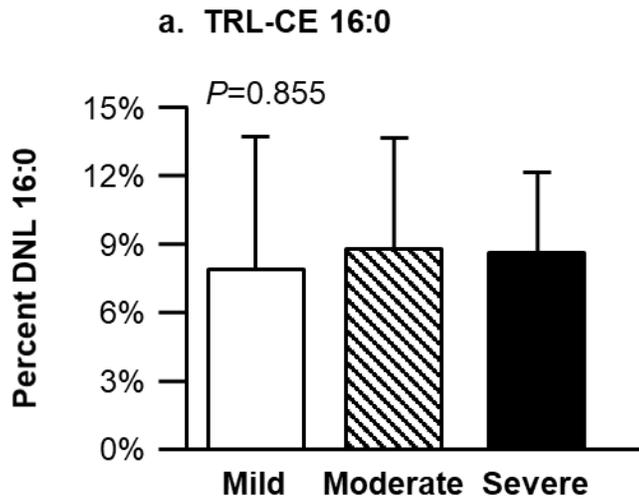
Legend: n=different for each panel. A bivariate Pearson correlation analysis with two-tail significance was performed to test the correlation between absolute DNL in liver-TAG and liver-CE with steatosis, inflammation, hepatocellular ballooning, fibrosis, AST, and ALT.

Figure 2.11: DNL in the TRL-TAG



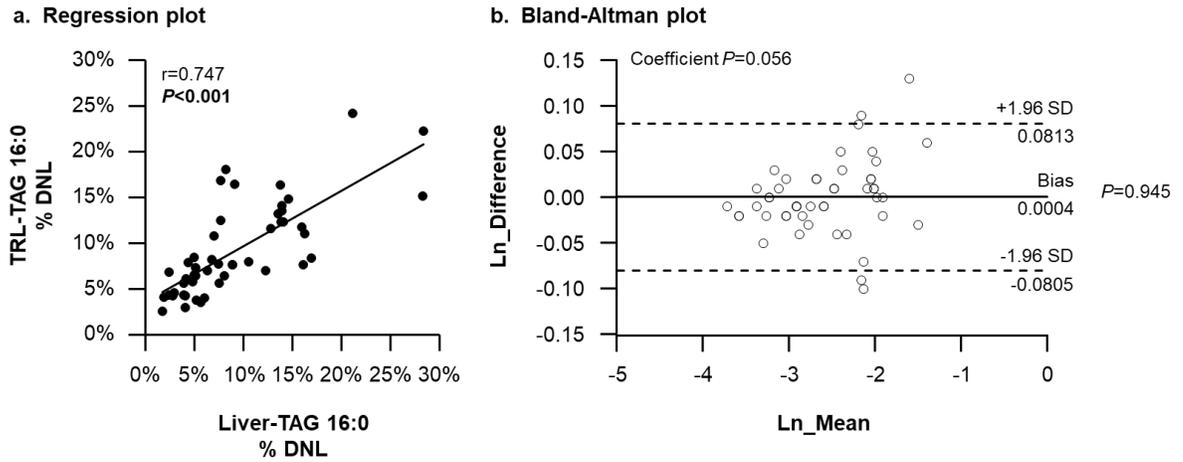
Legend: $n=49$. Data are reported in mean \pm SD. Absolute DNL was calculated by multiplying percent DNL with total TRL-TAG concentrations. One-way ANOVA was performed between the groups and the P -value was presented above each bar-graph. If significant, the Tukey post-hoc analysis was performed to test the significance of each group. * $P < 0.05$ compared to the mild group. † $P < 0.05$ compared to the moderate group.

Figure 2.12: DNL in the TRL-CE



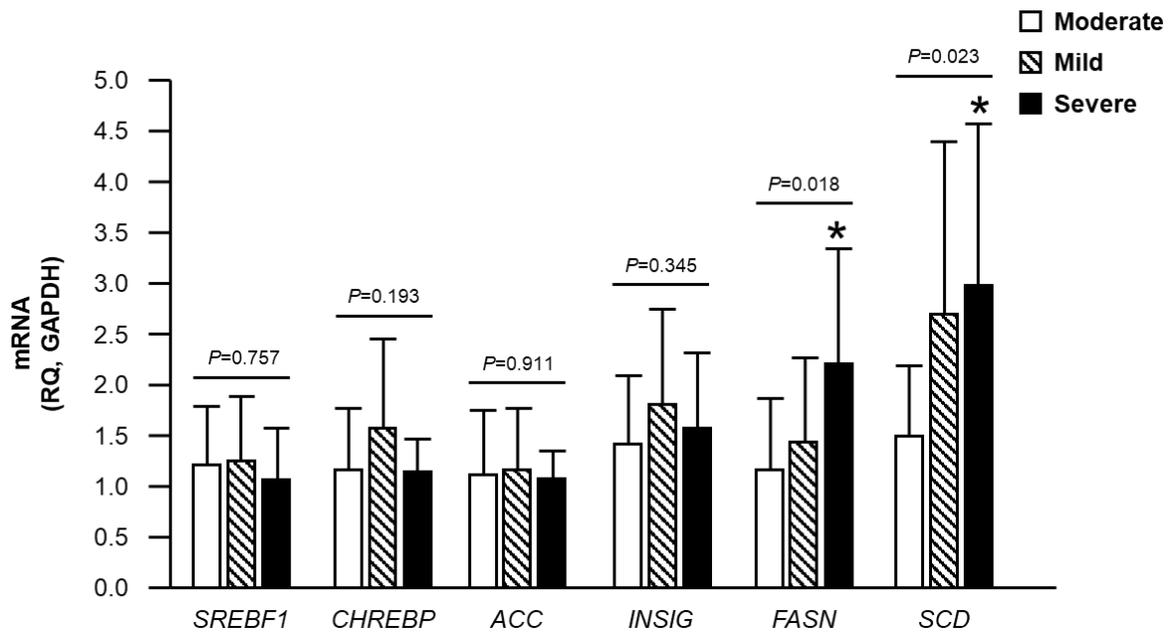
Legend: n=49. Data are reported in mean±SD. One-way ANOVA was performed between the groups and the *P*-value was presented above each bar-graph.

Figure 2.13: Relationships between liver and TRL percent DNL



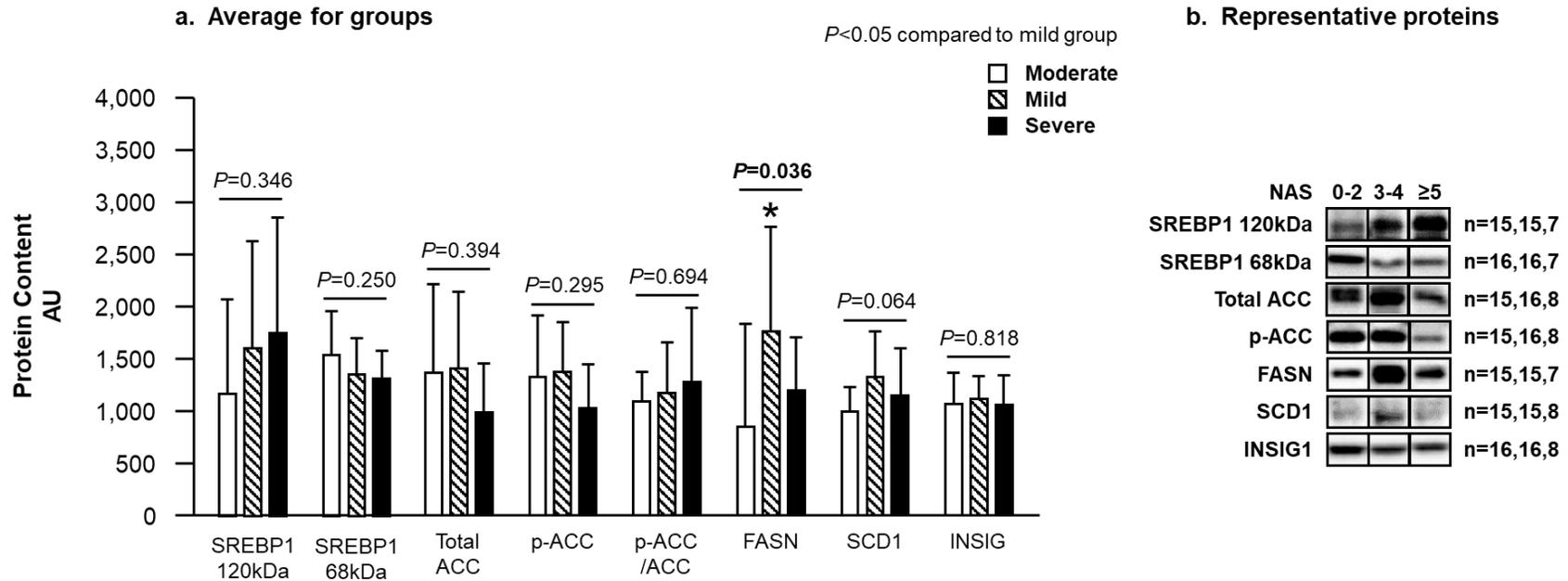
Legend: n=49. A linear regression analysis was performed to test the relationship between the DNL measured in the liver-TAG vs TRL-TAG (regression equation for liver-TAG %DNL = $0.007 + 0.918 \times \text{TRL-TAG \%DNL}$). A Bland-Altman plot was created by calculating the mean and differences between the logarithmic (Ln) values for DNL (liver-TAG and TRL-TAG). Bias was calculated as a mean of the Ln_Difference. A one-sample t-test was performed to calculate the *P*-value for the bias. A non-significant *P*-value suggests a strong relationship between both variables. Limits of agreement (LOA) were calculated by using the following formulas: upper limit: Bias+1.96*SD, lower limit: Bias-1.96*SD. A coefficient *P*-value for Ln_Mean was calculated by one-way ANOVA. A non-significant *P*-value for the coefficient suggests no bias between the variables.

Figure 2.14: mRNA expression of key genes involved in the DNL pathway



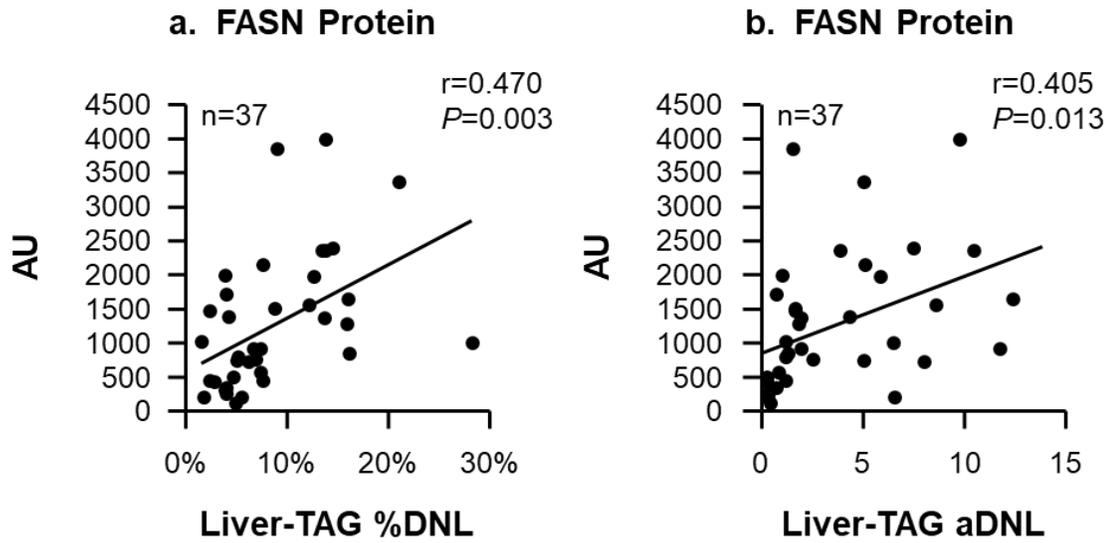
Legend: $n \geq 39$. Data are reported in mean \pm SD. One-way ANOVA was performed between the groups and the P -value was presented above each bar-graph. * $P < 0.05$ compared to the mild group. P -value for FASN and SCD between mild and moderate groups were $P = 0.07$.

Figure 2.15: Expression of key proteins involved in the DNL pathway



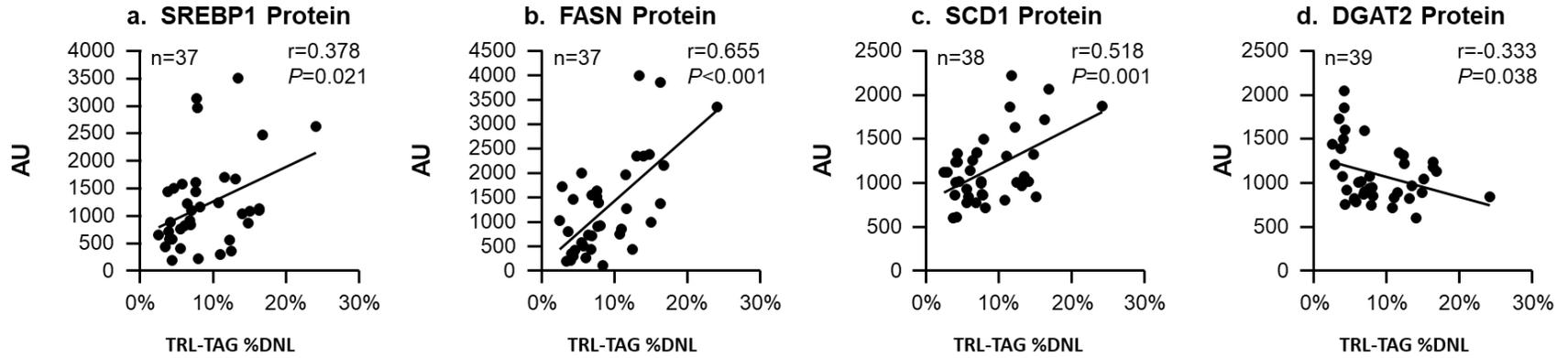
Legend: n≥37. Data are reported in mean±SD. One-way ANOVA was performed between the groups and the *P*-value was presented above each bar-graph. If significant, the Tukey post-hoc analysis was performed to test the significance of each group. **P*<0.05 compared to the mild group.

Figure 2.16: Correlation analysis of DNL and FASN



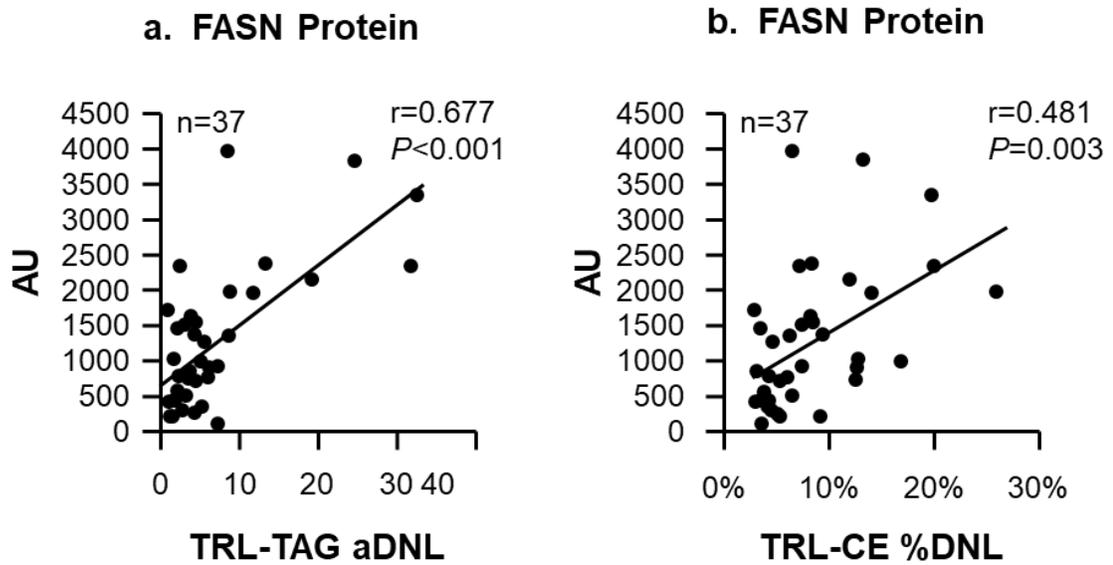
Legend: A bivariate Pearson correlation analysis with two-tail significance was performed to test the correlation between isotopically-labeled 16:0 DNL in liver-TAG and FASN protein.

Figure 2.17: Correlation analysis of labeled DNL vs DNL gene expression



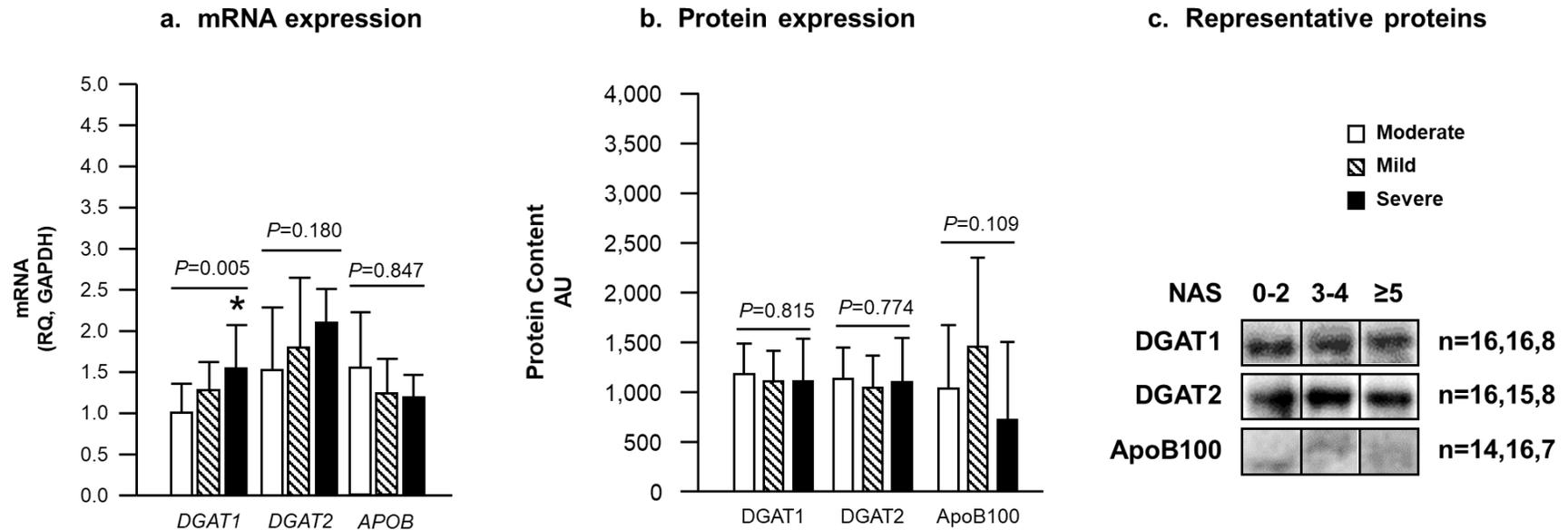
Legend: A bivariate Pearson correlation analysis with two-tail significance was performed to test the correlation between isotopically-labeled 16:0 DNL and DNL enzymes.

Figure 2.18: Correlation analysis of DNL and FASN



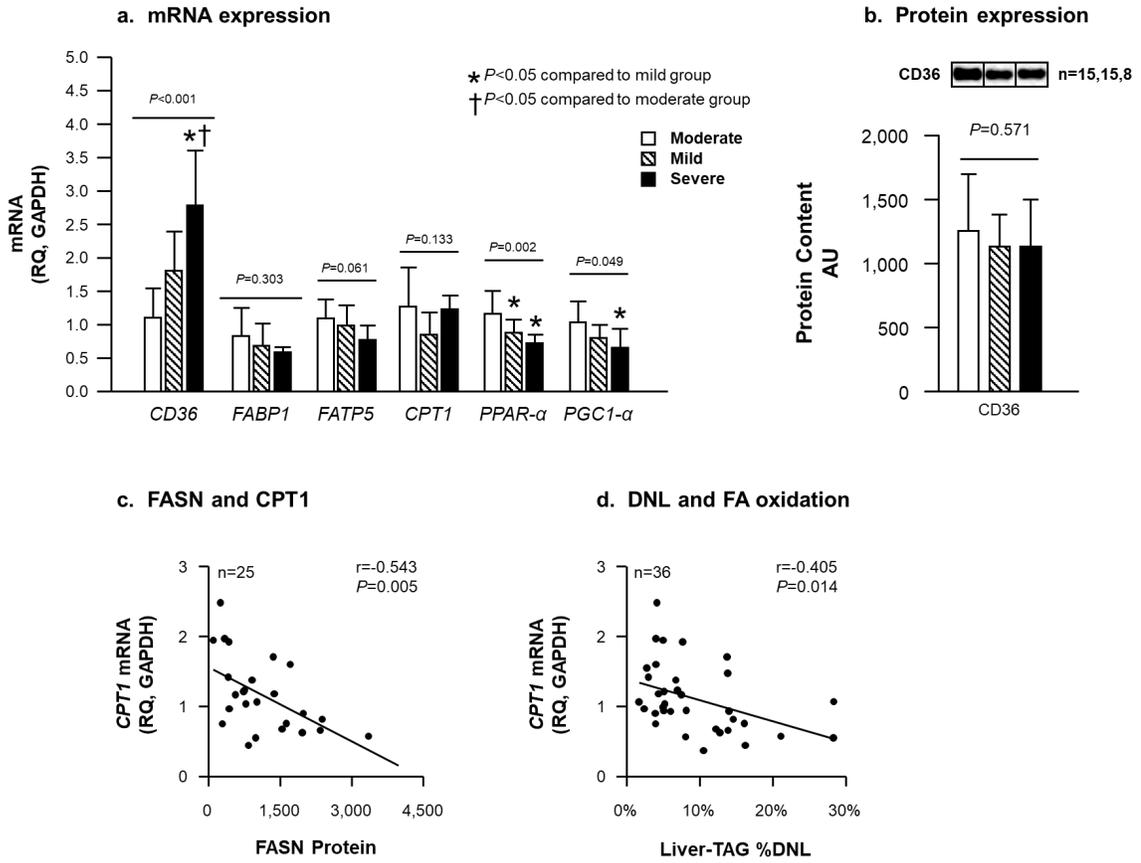
Legend: A bivariate Pearson correlation analysis with two-tail significance was performed to test the correlation between isotopically-labeled 16:0 DNL in TRL-TAG and FASN protein.

Figure 2.19: mRNA and protein expression of key enzymes involved in TAG metabolism



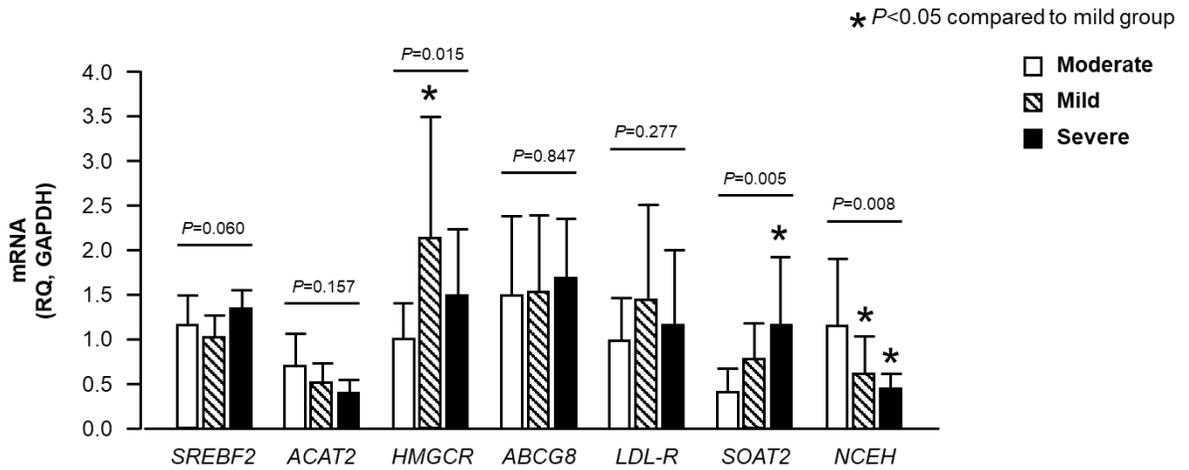
Legend: mRNA $n \geq 44$. Protein $n \geq 35$. Data are reported in mean \pm SD. One-way ANOVA was performed between the groups and the P -value was presented above each bar-graph. If significant, the Tukey post-hoc analysis was performed to test the significance of each group. * $P < 0.05$ compared to the mild group. For DGAT1 mRNA expression, the P -value between mild and moderate groups was $P = 0.102$.

Figure 2.20: mRNA and protein expression of key enzymes involved in FA transport and oxidation



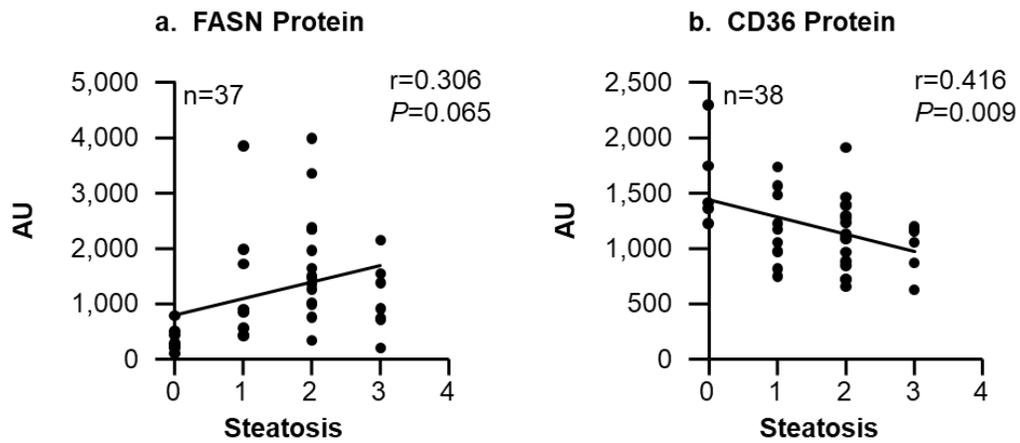
Legend a-b: mRNA $n \geq 33$. Protein $n = 38$. Data are reported in mean \pm SD. One-way ANOVA was performed between the groups and the P -value was presented above each bar-graph. For mRNA expression of *CD36*, P -value between the mild and moderate groups was $P = 0.065$. **Legend c-d:** A bivariate Pearson correlation analysis with two-tail significance was performed to test the correlation between liver-TAG percent DNL and *CPT1* mRNA expression.

Figure 2.21: mRNA expression of key genes involved in cholesterol metabolism



Legend: $n \geq 32$. Data are reported in mean \pm SD. One-way ANOVA was performed between the groups and the P -value was presented above each bar-graph. For mRNA expression of SOAT2, the P -value between the mild and the moderate group was $P=0.109$.

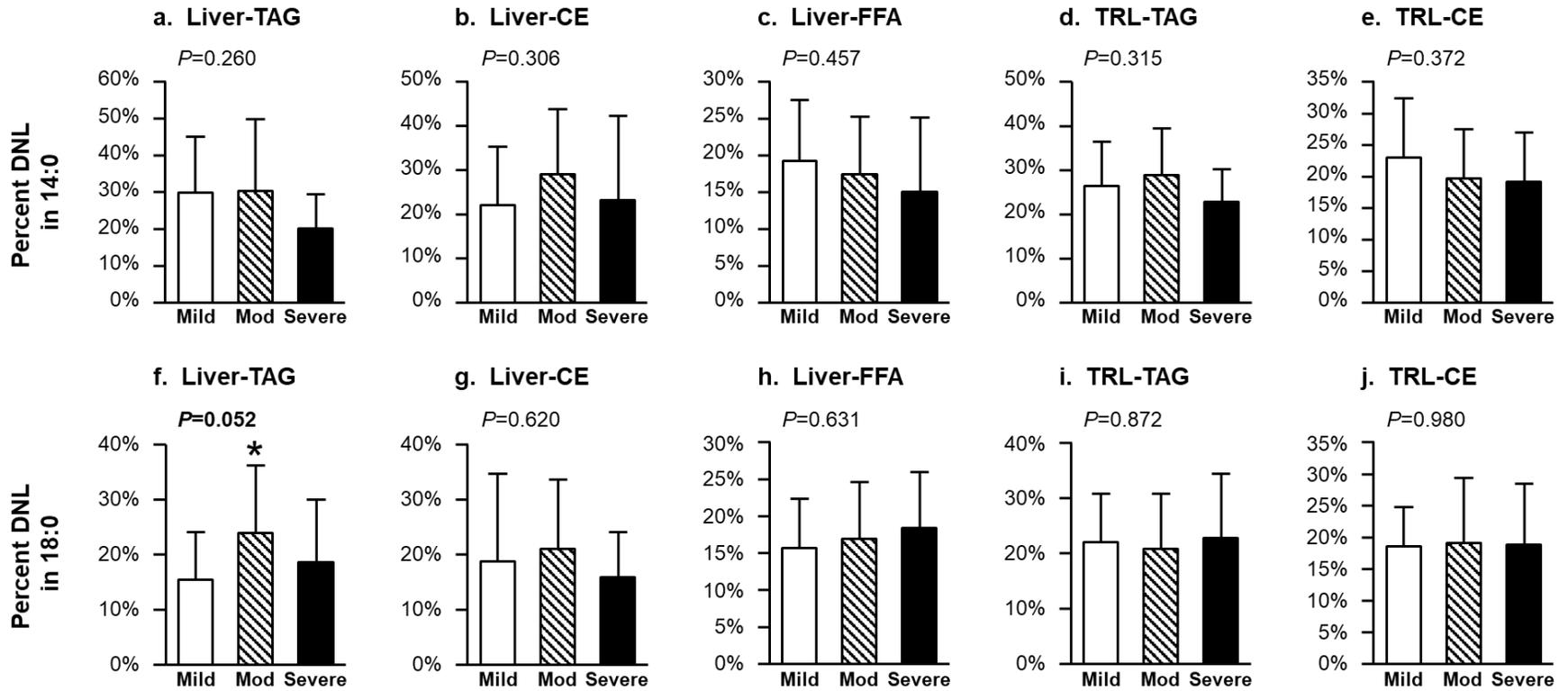
Figure 2.22: DNL FAs contribute more toward storage



Legend: n=different for each panel. A bivariate Pearson correlation analysis with two-tail significance was performed to test the correlation between steatosis grade and protein expression of FASN and CD36.

Figure 2.23: Percent DNL in other FA species

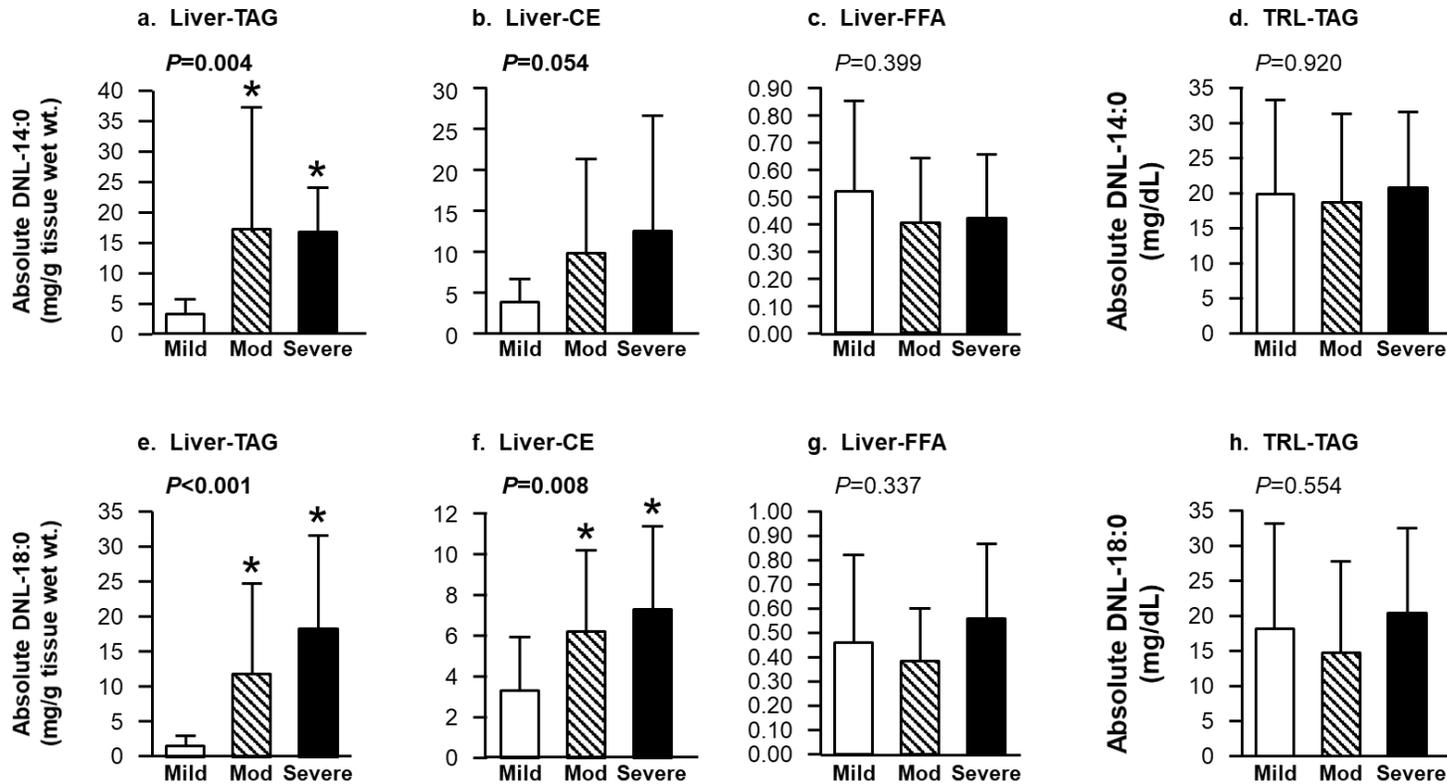
* $P < 0.05$ compared to mild group



Legend: n=49. Data are reported in mean±SD. One-way ANOVA was performed between the groups and the P -value was presented above each bar-graph. If significant, the Tukey post-hoc analysis was performed to test the significance of each group. * $P < 0.05$ compared to the mild group.

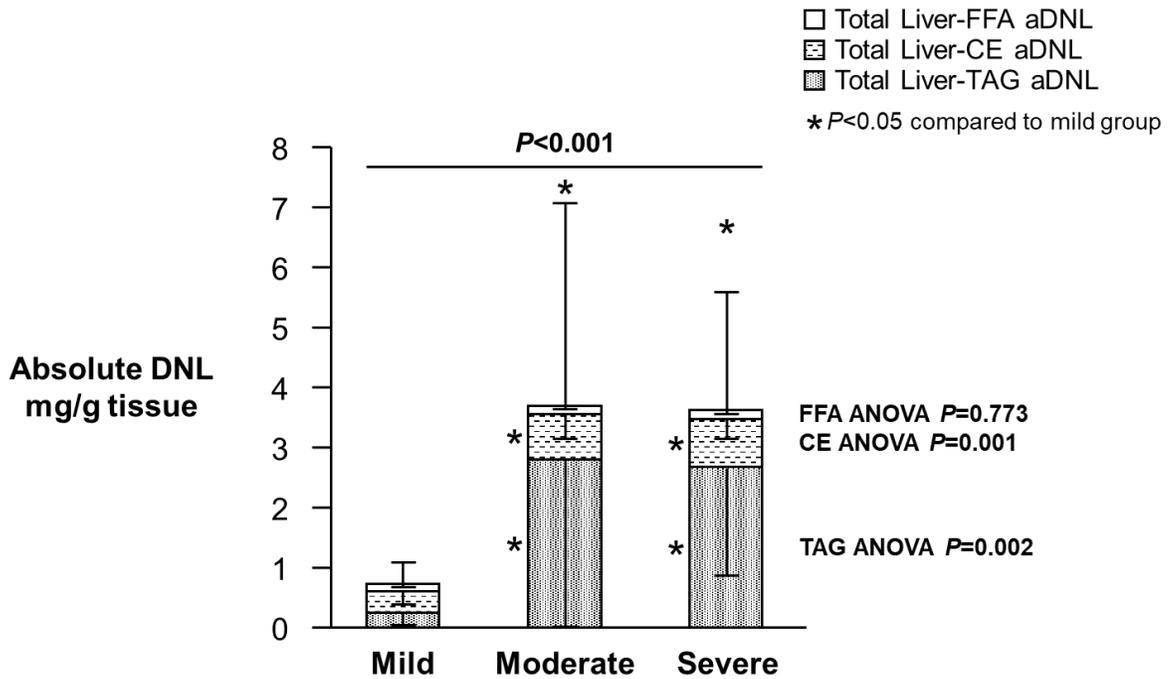
Figure 2.24: Absolute DNL in other FA species

* $P < 0.05$ compared to mild group



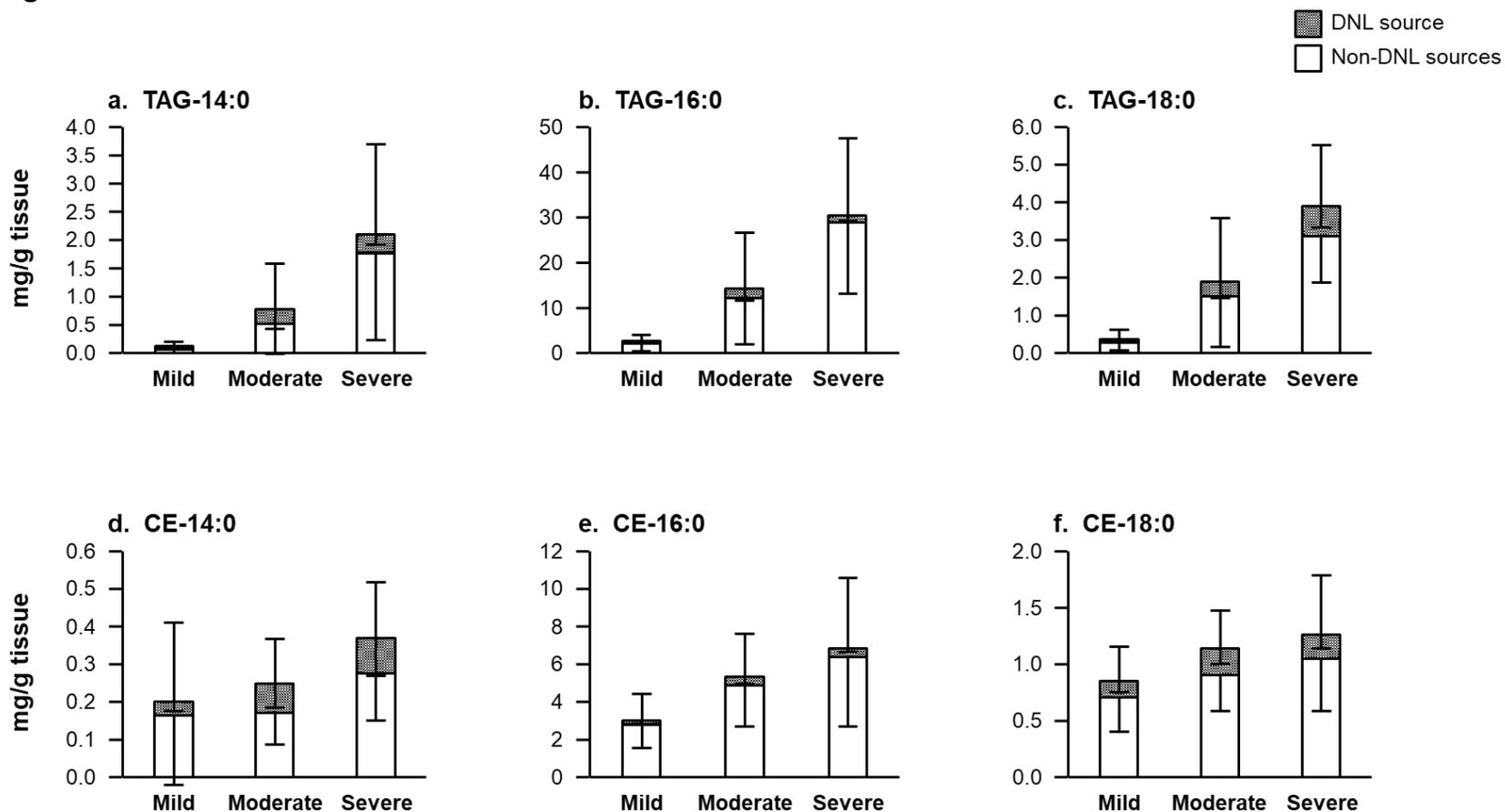
Legend: n=49. Data are reported in mean±SD. Absolute DNL was calculated by multiplying the percent DNL with the total concentration of the respective lipid class. One-way ANOVA was performed between the groups and the P -value was presented above each bar-graph. If significant, the Tukey post-hoc analysis was performed to test the significance of each group. * $P < 0.05$ compared to the mild group.

Figure 2.25: Total absolute DNL FAs in the liver



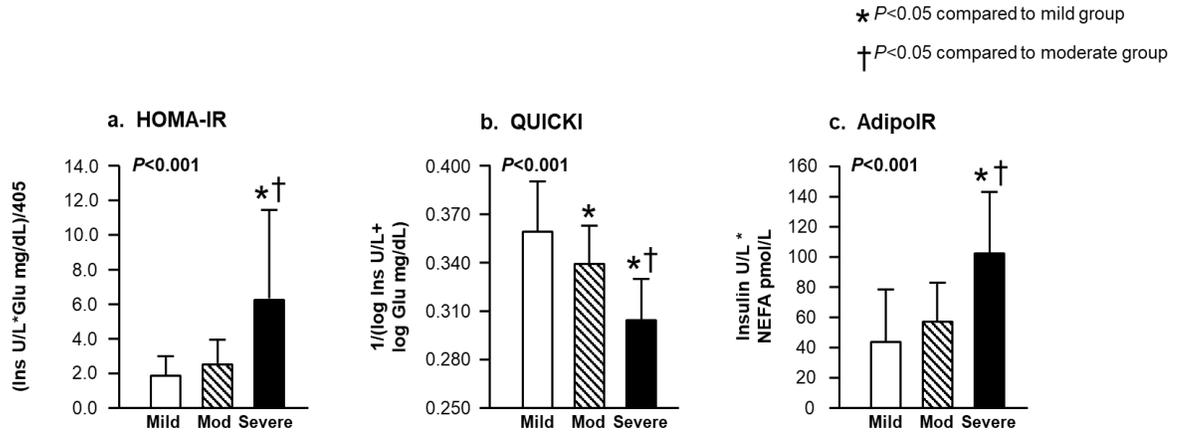
Legend: n=49. Data are reported in mean±SD. Absolute DNL was calculated by multiplying the percent DNL of each FA (14:0, 16:0, and 18:0) with the concentration of the specific FA (14:0, 16:0, and 18:0). The sum of absolute DNL of all three FAs was represented in the figure as total absolute DNL within each lipid class (TG, CE, and FFA). One-way ANOVA was performed between the groups and the P -value was presented above each bar-graph. If significant, the Tukey post-hoc analysis was performed to test the significance of each group. * $P < 0.05$ compared to the mild group.

Figure 2.26: Absolute DNL contribution in each FA



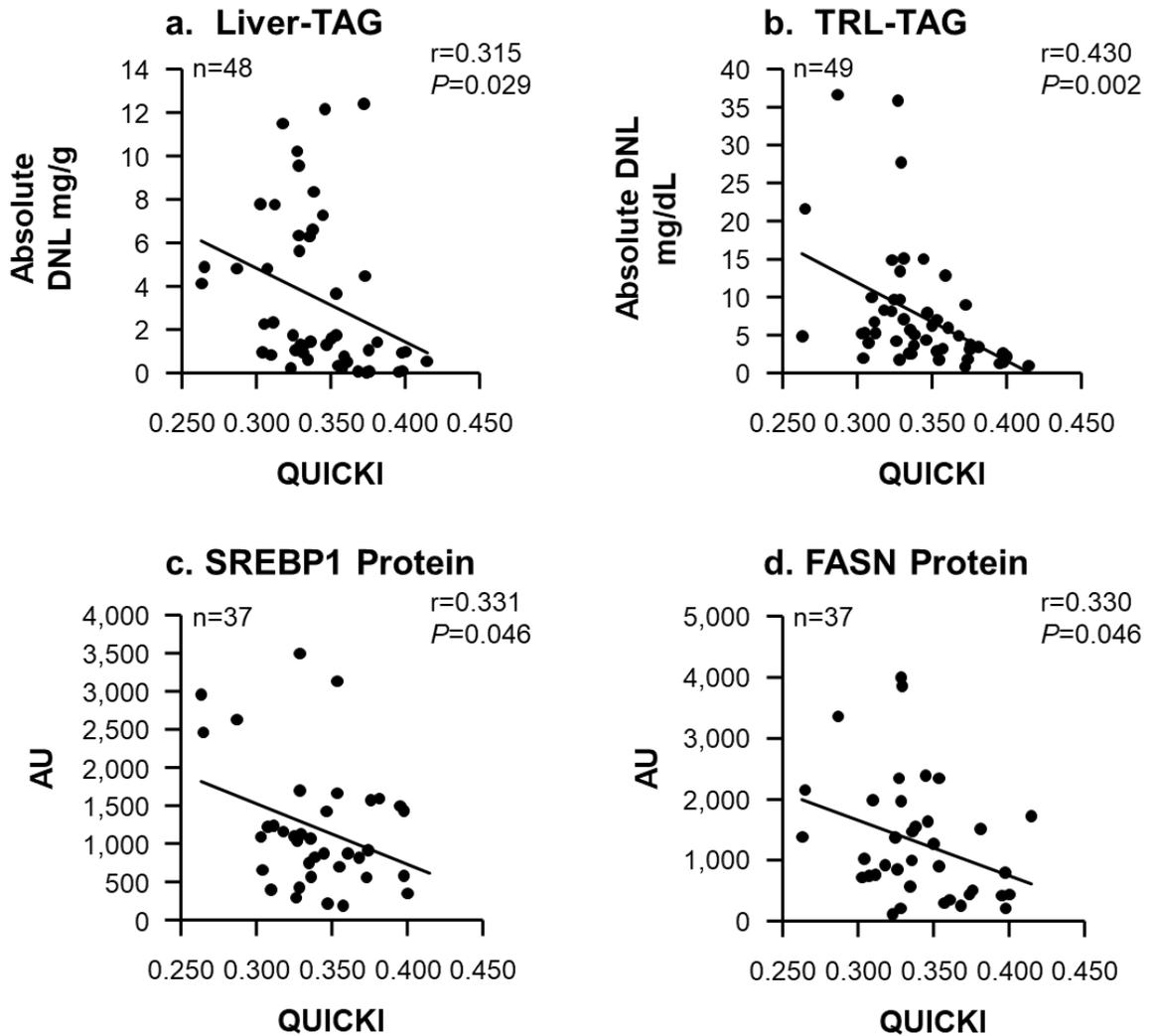
Legend: n=49. Data are reported in mean±SD. Absolute DNL was calculated by multiplying the percent DNL of each FA (14:0, 16:0, and 18:0) with the concentration of the specific FA (14:0, 16:0, and 18:0).

Figure 2.27: Insulin resistance in the severity of the disease



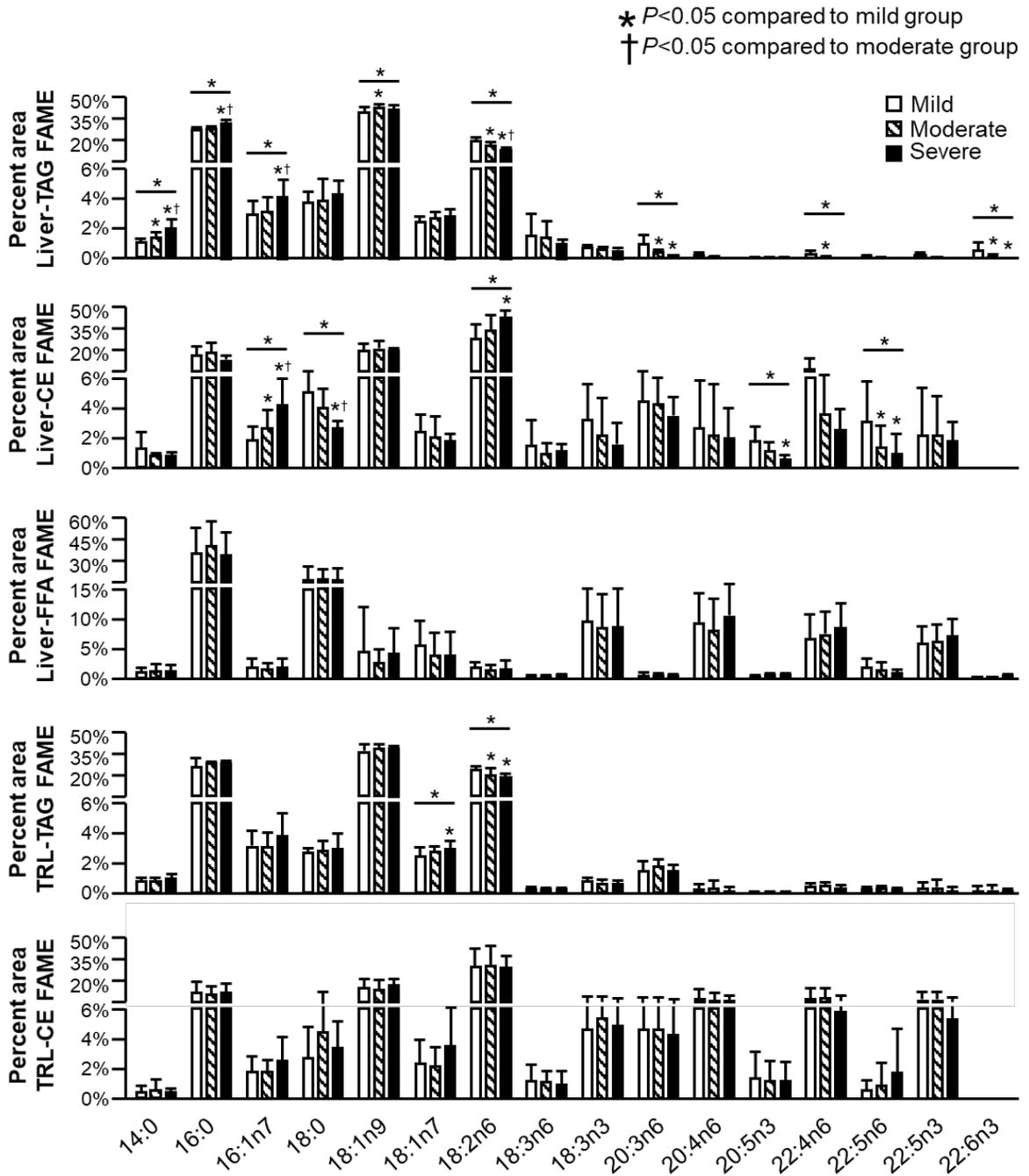
Legend: n=48. Data are reported in mean±SD. One-way ANOVA was performed between the groups and the P -value was presented above each bar-graph. If significant, the Tukey post-hoc analysis was performed to test the significance of each group. * $P < 0.05$ compared to the mild group, † $P < 0.05$ compared to the moderate group.

Figure 2.28: Insulin resistance, DNL, and DNL enzymes



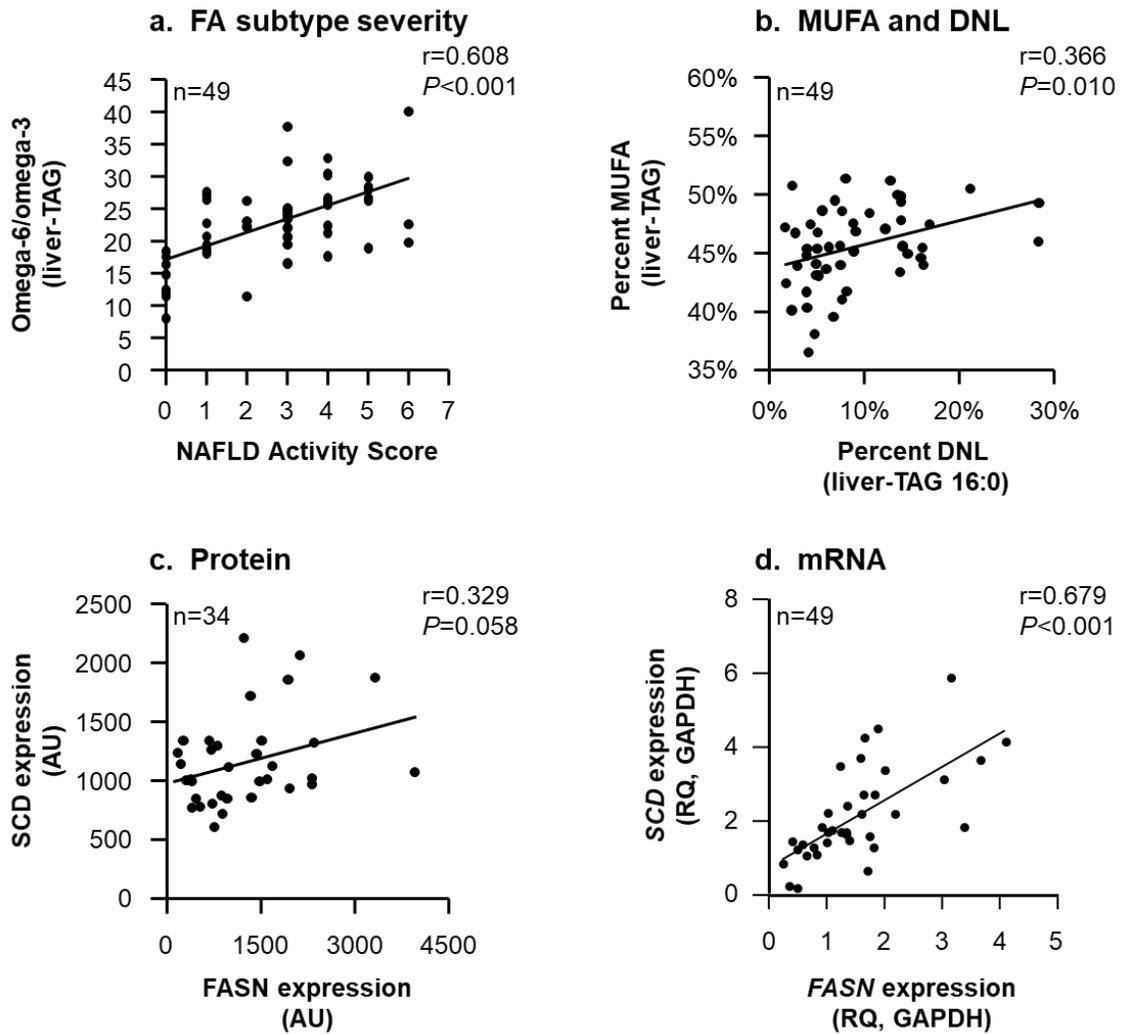
Legend: n=different for each panel. A bivariate Pearson correlation analysis with two-tail significance was performed to test the correlation between QUICKI and DNL in liver-TAG and TRL-TAG, and the protein expression of SREBP1 and FASN.

Figure 2.29: FA Composition in the liver and TRL lipids



Legend: n=49. Data are reported in mean±SD. One-way ANOVA was performed between the groups and the * sign above the line represents ANOVA $P < 0.05$. If significant, the Tukey post-hoc analysis was performed to test the significance of each group. * $P < 0.05$ compared to the mild group. † $P < 0.001$ compared to the moderate group.

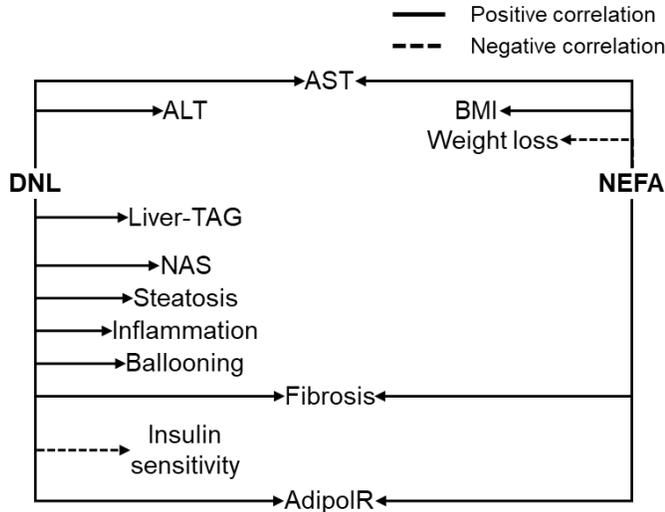
Figure 2.30: FA subclass, DNL enzymes, and NAFLD progression



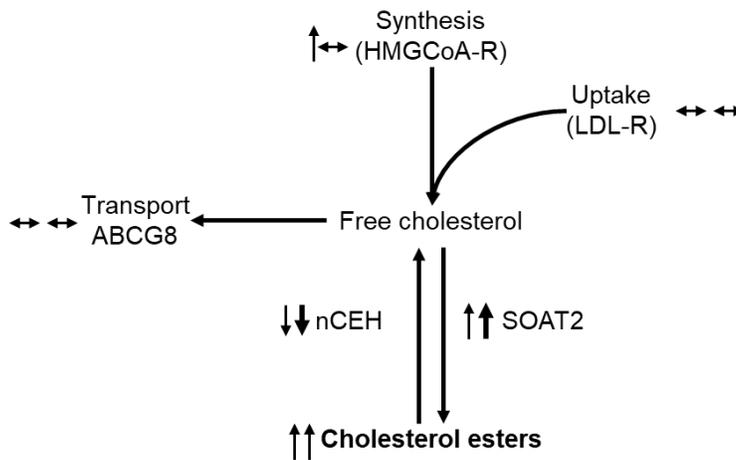
Legend: n=different for each panel. A bivariate Pearson correlation analysis with two-tail significance was performed to test the correlation between omega-6/omega-3 ratio and NAFLD activity score, percent MUFA and percent DNL, SCD and FASN protein expression, and SCD and FASN mRNA expression.

Figure 2.31: DNL vs NEFA associations with metabolic characteristics and cholesterol pathway in NAFLD

a. Impact of DNL vs NEFA on NAFLD progression

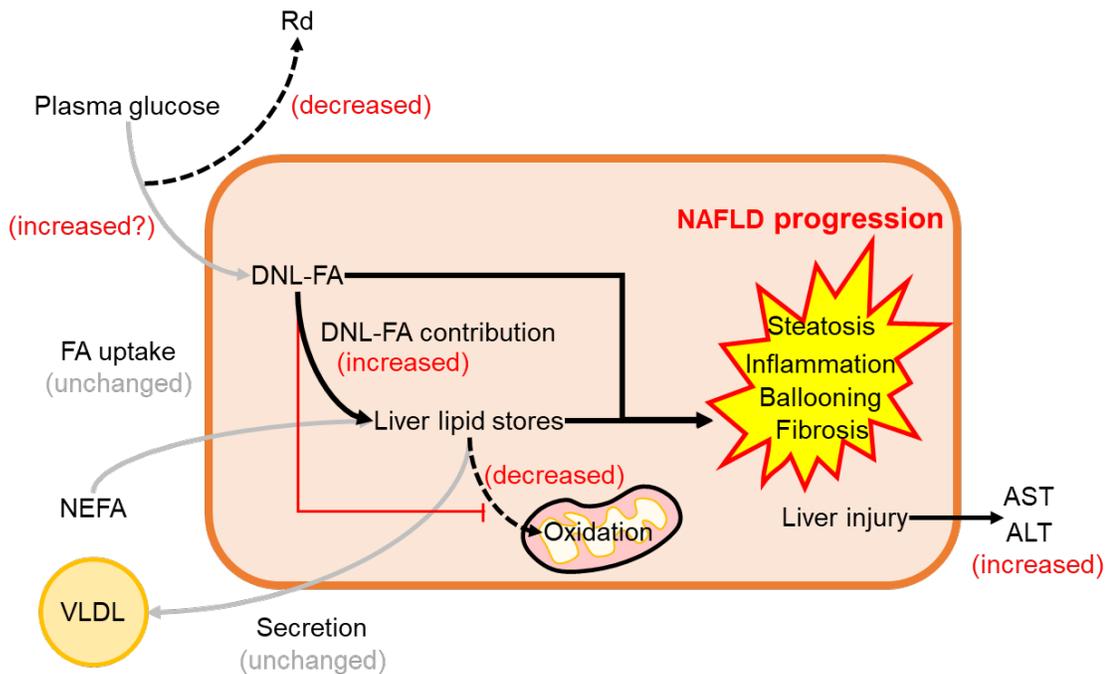


b. Changes in cholesterol pathway with NAFLD progression



Legend: a) represents correlations observed between FA synthesized via DNL vs NEFA. Solid lines represent positive correlation whereas dotted lines represent negative correlation. b) Represents the summary of data related to the cholesterol pathway with the severity of the disease. In the moderate group, increased liver-CE was due to increased synthesis, notable esterification, and decreased hydrolase activity, whereas, in the severe group, increased liver-CE was a result of increased esterification by SOAT2 and decreased NCEH activity. Arrows indicate the direction of change (↑ increased, ↓ decreased) and the line represents no change (↔). The first symbol represents the moderate group and the second symbol represents the severe group.

Figure 2.32: DNL contribution to NAFLD progression



Legend: Potential mechanism: In patients undergoing bariatric surgery, there appeared to be individuals with characteristics of NAFLD with varying severity. In all the groups including those who exhibited moderate or severe NAFLD characteristics, NEFA was elevated but was not different between the groups so did their VLDL secretion rates. Plasma glucose was not different between the groups and we postulated that in the mild group Rd was higher which allowed utilization of plasma glucose in the muscle resulting in normal plasma glucose whereas in the moderate and the severe groups, Rd was reduced due to insulin resistance and plasma glucose in the liver was increased by either GLUT2 activity or by gluconeogenesis for FA synthesis via DNL. Although the absolute DNL was higher in the moderate and the severe groups, the proportion of FA synthesized through DNL was yet low compared to the NEFA pool due to the fact that the patients were under negative energy balance. Therefore, the total contribution of FA from the DNL pathway was low in the liver lipid stores. Importantly, DNL inhibited the oxidation of FA by inhibiting the mitochondrial uptake of FA through the CPT1 enzyme and was linked directly to the progression of individual NAFLD characteristics (steatosis, inflammation, ballooning, and fibrosis) resulting in increased liver enzymes (AST and ALT) in the plasma.

REFERENCES

1. Browning, J. D., & Horton, J. D. (2004). Molecular mediators of hepatic steatosis and liver injury. *J Clin Invest*, *114*(2), 147-152. doi:10.1172/JCI22422. **PMC449757**.
2. Adams, L. A., Lymp, J. F., St Sauver, J., Sanderson, S. O., Lindor, K. D., Feldstein, A., & Angulo, P. (2005). The natural history of nonalcoholic fatty liver disease: a population-based cohort study. *Gastroenterology*, *129*(1), 113-121. doi:10.1053/j.gastro.2005.04.014. **PMID: 16012941**.
3. Rinella, M. E. (2015). Nonalcoholic fatty liver disease: a systematic review. *JAMA*, *313*(22), 2263-2273. doi:10.1001/jama.2015.5370. **PMID: 26057287**.
4. Younossi, Z. M., Koenig, A. B., Abdelatif, D., Fazel, Y., Henry, L., & Wymer, M. (2016). Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology*, *64*(1), 73-84. doi:10.1002/hep.28431. **PMID: 26707365**.
5. Calzadilla Bertot, L., & Adams, L. A. (2016). The Natural course of non-alcoholic fatty liver disease. *Int J Mol Sci*, *17*(5). doi:10.3390/ijms17050774. **PMC4881593**.
6. Ray, K. (2013). NAFLD-the next global epidemic. *Nat Rev Gastroenterol Hepatol*, *10*(11), 621. doi:10.1038/nrgastro.2013.197. **PMID: 24185985**.
7. Younossi, Z., Stepanova, M., Ong, J. P., Jacobson, I. M., Bugianesi, E., Duseja, A., Eguchi, Y., Wong, V. W., Negro, F., Yilmaz, Y., Romero-Gomez, M., George, J., Ahmed, A., Wong, R., Younossi, I., Ziayee, M., Afendy, A., & Global Nonalcoholic Steatohepatitis, C. (2019). Nonalcoholic steatohepatitis is the fastest growing cause of hepatocellular carcinoma in liver transplant candidates. *Clin Gastroenterol Hepatol*, *17*(4), 748-755 e743. doi:10.1016/j.cgh.2018.05.057. **PMID: 29908364**.
8. Eslam, M., Valenti, L., & Romeo, S. (2018). Genetics and epigenetics of NAFLD and NASH: Clinical impact. *J Hepatol*, *68*(2), 268-279. doi:10.1016/j.jhep.2017.09.003. **PMID: 29122391**.
9. Lambert, J. E., Ramos-Roman, M. A., Browning, J. D., & Parks, E. J. (2014). Increased de novo lipogenesis is a distinct characteristic of individuals with nonalcoholic fatty liver disease. *Gastroenterology*, *146*(3), 726-735. doi:10.1053/j.gastro.2013.11.049. **PMC6276362**.
10. Donnelly, K. L., Smith, C. I., Schwarzenberg, S. J., Jessurun, J., Boldt, M. D., & Parks, E. J. (2005). Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J Clin Invest*, *115*(5), 1343-1351. doi:10.1172/JCI23621. **PMC1087172**.
11. Schwarz, J. M., Clearfield, M., & Mulligan, K. (2017). Conversion of sugar to fat: Is hepatic de novo lipogenesis leading to metabolic syndrome and associated chronic diseases? *J Am Osteopath Assoc*, *117*(8), 520-527. doi:10.7556/jaoa.2017.102. **PMID: 28759094**.
12. Chiu, S., Mulligan, K., & Schwarz, J. M. (2018). Dietary carbohydrates and fatty liver disease: de novo lipogenesis. *Curr Opin Clin Nutr Metab Care*,

- 21(4), 277-282. doi:10.1097/MCO.0000000000000469. **PMID: 29697539.**
13. Schwarz, J. M., Linfoot, P., Dare, D., & Aghajanian, K. (2003). Hepatic de novo lipogenesis in normoinsulinemic and hyperinsulinemic subjects consuming high-fat, low-carbohydrate and low-fat, high-carbohydrate isoenergetic diets. *Am J Clin Nutr*, 77(1), 43-50. doi:10.1093/ajcn/77.1.43. **PMID: 12499321.**
 14. Silva, J. C. P., Marques, C., Martins, F. O., Viegas, I., Tavares, L., Macedo, M. P., & Jones, J. G. (2019). Determining contributions of exogenous glucose and fructose to de novo fatty acid and glycerol synthesis in liver and adipose tissue. *Metab Eng*, 56, 69-76. doi:10.1016/j.ymben.2019.08.018. **PMID: 31473320.**
 15. Smith, G. I., Shankaran, M., Yoshino, M., Schweitzer, G. G., Chondronikola, M., Beals, J. W., Okunade, A. L., Patterson, B. W., Nyangau, E., Field, T., Sirlin, C. B., Talukdar, S., Hellerstein, M. K., & Klein, S. (2020). Insulin resistance drives hepatic de novo lipogenesis in nonalcoholic fatty liver disease. *J Clin Invest*, 130(3), 1453-1460. doi:10.1172/JCI134165. **PMC7269561.**
 16. Dif, N., Euthine, V., Gonnet, E., Laville, M., Vidal, H., & Lefai, E. (2006). Insulin activates human sterol-regulatory-element-binding protein-1c (SREBP-1c) promoter through SRE motifs. *Biochem J*, 400(1), 179-188. doi:10.1042/BJ20060499. **PMC1635455.**
 17. Liang, G., Yang, J., Horton, J. D., Hammer, R. E., Goldstein, J. L., & Brown, M. S. (2002). Diminished hepatic response to fasting/refeeding and liver X receptor agonists in mice with selective deficiency of sterol regulatory element-binding protein-1c. *J Biol Chem*, 277(11), 9520-9528. doi:10.1074/jbc.M111421200. **PMID: 11782483.**
 18. Matsuzaka, T., & Shimano, H. (2013). Insulin-dependent and -independent regulation of sterol regulatory element-binding protein-1c. *J Diabetes Investig*, 4(5), 411-412. doi:10.1111/jdi.12098. **PMC4025107.**
 19. Jelenik, T., Kaul, K., Sequaris, G., Flogel, U., Phielix, E., Kotzka, J., Knebel, B., Fahlbusch, P., Horbelt, T., Lehr, S., Reinbeck, A. L., Muller-Wieland, D., Esposito, I., Shulman, G. I., Szendroedi, J., & Roden, M. (2017). Mechanisms of insulin resistance in primary and secondary nonalcoholic fatty liver. *Diabetes*, 66(8), 2241-2253. doi:10.2337/db16-1147. **PMC5521856.**
 20. Min, H. K., Kapoor, A., Fuchs, M., Mirshahi, F., Zhou, H., Maher, J., Kellum, J., Warnick, R., Contos, M. J., & Sanyal, A. J. (2012). Increased hepatic synthesis and dysregulation of cholesterol metabolism is associated with the severity of nonalcoholic fatty liver disease. *Cell Metab*, 15(5), 665-674. doi:10.1016/j.cmet.2012.04.004. **PMC3361911.**
 21. Puri, P., Baillie, R. A., Wiest, M. M., Mirshahi, F., Choudhury, J., Cheung, O., Sargeant, C., Contos, M. J., & Sanyal, A. J. (2007). A lipidomic analysis of nonalcoholic fatty liver disease. *Hepatology*, 46(4), 1081-1090. doi:10.1002/hep.21763. **PMID: 17654743.**

22. Syed-Abdul, M. M., Parks, E. J., Gaballah, A. H., Bingham, K., Hammoud, G. M., Kemble, G., Buckley, D., McCulloch, W., & Manrique-Acevedo, C. (2020). Fatty acid synthase inhibitor TVB-2640 reduces hepatic de novo lipogenesis in males with metabolic abnormalities. *Hepatology*, 72(1), 103-118. doi:10.1002/hep.31000. **PMID: 31630414**.
23. Kim, C. W., Addy, C., Kusunoki, J., Anderson, N. N., Deja, S., Fu, X., Burgess, S. C., Li, C., Ruddy, M., Chakravarthy, M., Previs, S., Milstein, S., Fitzgerald, K., Kelley, D. E., & Horton, J. D. (2017). Acetyl CoA carboxylase inhibition reduces hepatic steatosis but elevates plasma triglycerides in mice and humans: A bedside to bench investigation. *Cell Metab*, 26(2), 394-406 e396. doi:10.1016/j.cmet.2017.07.009. **PMC5603267**.
24. Lawitz, E. J., Coste, A., Poordad, F., Alkhoury, N., Loo, N., McColgan, B. J., Tarrant, J. M., Nguyen, T., Han, L., Chung, C., Ray, A. S., McHutchison, J. G., Subramanian, G. M., Myers, R. P., Middleton, M. S., Sirlin, C., Loomba, R., Nyangau, E., Fitch, M., Li, K., & Hellerstein, M. (2018). Acetyl-CoA carboxylase inhibitor GS-0976 for 12 weeks reduces hepatic de novo lipogenesis and steatosis in patients with nonalcoholic steatohepatitis. *Clin Gastroenterol Hepatol*, 16(12), 1983-1991 e1983. doi:10.1016/j.cgh.2018.04.042. **PMID: 29705265**.
25. Loomba, R., Kayali, Z., Nouredin, M., Ruane, P., Lawitz, E. J., Bennett, M., Wang, L., Harting, E., Tarrant, J. M., McColgan, B. J., Chung, C., Ray, A. S., Subramanian, G. M., Myers, R. P., Middleton, M. S., Lai, M., Charlton, M., & Harrison, S. A. (2018). GS-0976 reduces hepatic steatosis and fibrosis markers in patients with nonalcoholic fatty liver disease. *Gastroenterology*, 155(5), 1463-1473 e1466. doi:10.1053/j.gastro.2018.07.027. **PMC6318218**.
26. Stiede, K., Miao, W., Blanchette, H. S., Beysen, C., Harriman, G., Harwood, H. J., Jr., Kelley, H., Kapeller, R., Schmalbach, T., & Westlin, W. F. (2017). Acetyl-coenzyme A carboxylase inhibition reduces de novo lipogenesis in overweight male subjects: A randomized, double-blind, crossover study. *Hepatology*, 66(2), 324-334. doi:10.1002/hep.29246. **PMC5599970**.
27. Bergman, A., Carvajal-Gonzalez, S., Tarabar, S., Saxena, A. R., Esler, W. P., & Amin, N. B. (2020). Safety, tolerability, pharmacokinetics, and pharmacodynamics of a liver-targeting acetyl-CoA carboxylase inhibitor (PF-05221304): A three-part randomized phase 1 study. *Clin Pharmacol Drug Dev*, 9(4), 514-526. doi:10.1002/cpdd.782. **PMC7317421**.
28. Mechanick, J. I., Apovian, C., Brethauer, S., Garvey, W. T., Joffe, A. M., Kim, J., Kushner, R. F., Lindquist, R., Pessah-Pollack, R., Seger, J., Urman, R. D., Adams, S., Cleek, J. B., Correa, R., Figaro, M. K., Flanders, K., Grams, J., Hurley, D. L., Kothari, S., Seger, M. V., & Still, C. D. (2020). Clinical practice guidelines for the perioperative nutrition, metabolic, and nonsurgical support of patients undergoing bariatric procedures - 2019 update: cosponsored by American Association of Clinical Endocrinologists/American College of Endocrinology, The Obesity

- Society, American Society for Metabolic & Bariatric Surgery, Obesity Medicine Association, and American Society of Anesthesiologists. *Surg Obes Relat Dis*, 16(2), 175-247. doi:10.1016/j.soard.2019.10.025. **PMID: 31917200.**
29. Mechanick, J. I., Apovian, C., Brethauer, S., Timothy Garvey, W., Joffe, A. M., Kim, J., Kushner, R. F., Lindquist, R., Pessah-Pollack, R., Seger, J., Urman, R. D., Adams, S., Cleek, J. B., Correa, R., Figaro, M. K., Flanders, K., Grams, J., Hurley, D. L., Kothari, S., Seger, M. V., & Still, C. D. (2020). Clinical practice guidelines for the perioperative nutrition, metabolic, and nonsurgical support of patients undergoing bariatric procedures - 2019 update: Cosponsored by American Association of Clinical Endocrinologists/American College of Endocrinology, The Obesity Society, American Society for Metabolic and Bariatric Surgery, Obesity Medicine Association, and American Society of Anesthesiologists. *Obesity (Silver Spring)*, 28(4), O1-O58. doi:10.1002/oby.22719. **PMID: 32202076.**
 30. Mechanick, J. I., Apovian, C., Brethauer, S., Garvey, W. T., Joffe, A. M., Kim, J., Kushner, R. F., Lindquist, R., Pessah-Pollack, R., Seger, J., Urman, R. D., Adams, S., Cleek, J. B., Correa, R., Figaro, M. K., Flanders, K., Grams, J., Hurley, D. L., Kothari, S., Seger, M. V., & Still, C. D. (2019). Clinical practice guidelines for the perioperative nutrition, metabolic, and nonsurgical support of patients undergoing bariatric procedures - 2019 update: Cosponsored by American Association of Clinical Endocrinologists/American College of Endocrinology, the Obesity Society, American Society for Metabolic & Bariatric Surgery, Obesity Medicine Association, and American Society of Anesthesiologists - Executive summary. *Endocr Pract*, 25(12), 1346-1359. doi:10.4158/GL-2019-0406. **PMID: 31682518.**
 31. Udelsman, B. V., Corey, K. E., Lindvall, C., Gee, D. W., Meireles, O. R., Hutter, M. M., Chang, D. C., & Witkowski, E. R. (2019). Risk factors and prevalence of liver disease in review of 2557 routine liver biopsies performed during bariatric surgery. *Surg Obes Relat Dis*, 15(6), 843-849. doi:10.1016/j.soard.2019.01.035. **PMID: 31014948.**
 32. Syed-Abdul, M. M., Hu, Q., Jacome-Sosa, M., Padilla, J., Manrique-Acevedo, C., Heimowitz, C., & Parks, E. J. (2018). Effect of carbohydrate restriction-induced weight loss on aortic pulse wave velocity in overweight men and women. *Appl Physiol Nutr Metab*, 43(12), 1247-1256. doi:10.1139/apnm-2018-0113. **PMID: 29746789.**
 33. Brunt, E. M., Kleiner, D. E., Wilson, L. A., Belt, P., Neuschwander-Tetri, B. A., & Network, N. C. R. (2011). Nonalcoholic fatty liver disease (NAFLD) activity score and the histopathologic diagnosis in NAFLD: distinct clinicopathologic meanings. *Hepatology*, 53(3), 810-820. doi:10.1002/hep.24127. **PMC3079483.**
 34. Moore, M. P., Cunningham, R. P., Kelty, T. J., Boccardi, L. R., Nguyen, N. Y., Booth, F. W., & Rector, R. S. (2020). Ketogenic diet in combination with voluntary exercise impacts markers of hepatic metabolism and

- oxidative stress in male and female Wistar rats. *Appl Physiol Nutr Metab*, 45(1), 35-44. doi:10.1139/apnm-2019-0042. **PMID: 31116955**.
35. Rector, R. S., Thyfault, J. P., Morris, R. T., Laye, M. J., Borengasser, S. J., Booth, F. W., & Ibdah, J. A. (2008). Daily exercise increases hepatic fatty acid oxidation and prevents steatosis in Otsuka Long-Evans Tokushima Fatty rats. *Am J Physiol Gastrointest Liver Physiol*, 294(3), G619-626. doi:10.1152/ajpgi.00428.2007. **PMID: 18174272**.
36. Sheldon, R. D., Meers, G. M., Morris, E. M., Linden, M. A., Cunningham, R. P., Ibdah, J. A., Thyfault, J. P., Laughlin, M. H., & Rector, R. S. (2019). eNOS deletion impairs mitochondrial quality control and exacerbates Western diet-induced NASH. *Am J Physiol Endocrinol Metab*, 317(4), E605-E616. doi:10.1152/ajpendo.00096.2019. **PMC6842915**.
37. Folch, J., Lees, M., & Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem*, 226(1), 497-509. **PMID: 13428781**.
38. Barrows, B. R., & Parks, E. J. (2006). Contributions of different fatty acid sources to very low-density lipoprotein-triacylglycerol in the fasted and fed states. *J Clin Endocrinol Metab*, 91(4), 1446-1452. doi:10.1210/jc.2005-1709. **PMID: 16449340**.
39. Hellerstein, M. K., & Neese, R. A. (1992). Mass isotopomer distribution analysis: a technique for measuring biosynthesis and turnover of polymers. *Am J Physiol*, 263(5 Pt 1), E988-1001. doi:10.1152/ajpendo.1992.263.5.E988. **PMID: 1443132**.
40. Bederman, I. R., Foy, S., Chandramouli, V., Alexander, J. C., & Previs, S. F. (2009). Triglyceride synthesis in epididymal adipose tissue: contribution of glucose and non-glucose carbon sources. *J Biol Chem*, 284(10), 6101-6108. doi:10.1074/jbc.M808668200. **PMC2649080**.
41. Patton, G. M., & Lowenstein, J. M. (1979). Measurements of fatty acid synthesis by incorporation of deuterium from deuterated water. *Biochemistry*, 18(14), 3186-3188. doi:10.1021/bi00581a042. **PMID: 465462**.
42. Parks, E. J., Skokan, L. E., Timlin, M. T., & Dingfelder, C. S. (2008). Dietary sugars stimulate fatty acid synthesis in adults. *J Nutr*, 138(6), 1039-1046. doi:10.1093/jn/138.6.1039. **PMC2546703**.
43. Gasteyer, C., Larsen, T. M., Vercruyse, F., & Astrup, A. (2008). Effect of a dietary-induced weight loss on liver enzymes in obese subjects. *Am J Clin Nutr*, 87(5), 1141-1147. doi:10.1093/ajcn/87.5.1141. **PMID: 18469232**.
44. Jhaveri, M. A., & Anderson, J. W. (2010). Sequential changes of serum aminotransferase levels in severely obese patients after losing weight through enrollment in a behavioral weight loss program. *Postgrad Med*, 122(4), 206-212. doi:10.3810/pgm.2010.07.2188. **PMID: 20675984**.
45. Huang, Y., Jin, Q., Su, M., Ji, F., Wang, N., Zhong, C., Jiang, Y., Liu, Y., Zhang, Z., Yang, J., Wei, L., Chen, T., & Li, B. (2017). Leptin promotes the migration and invasion of breast cancer cells by upregulating ACAT2.

- Cell Oncol (Dordr)*, 40(6), 537-547. doi:10.1007/s13402-017-0342-8.
PMID: 28770546.
46. Silha, J. V., Krsek, M., Skrha, J. V., Sucharda, P., Nyomba, B. L., & Murphy, L. J. (2003). Plasma resistin, adiponectin and leptin levels in lean and obese subjects: correlations with insulin resistance. *Eur J Endocrinol*, 149(4), 331-335. doi:10.1530/eje.0.1490331. **PMID: 14514348.**
 47. Polyzos, S. A., Aronis, K. N., Kountouras, J., Raptis, D. D., Vasiloglou, M. F., & Mantzoros, C. S. (2016). Circulating leptin in non-alcoholic fatty liver disease: a systematic review and meta-analysis. *Diabetologia*, 59(1), 30-43. doi:10.1007/s00125-015-3769-3. **PMID: 26407715.**
 48. Alger, H. M., Brown, J. M., Sawyer, J. K., Kelley, K. L., Shah, R., Wilson, M. D., Willingham, M. C., & Rudel, L. L. (2010). Inhibition of acyl-coenzyme A:cholesterol acyltransferase 2 (ACAT2) prevents dietary cholesterol-associated steatosis by enhancing hepatic triglyceride mobilization. *J Biol Chem*, 285(19), 14267-14274. doi:10.1074/jbc.M110.118422.
PMC2863169.
 49. Fabbrini, E., Mohammed, B. S., Magkos, F., Korenblat, K. M., Patterson, B. W., & Klein, S. (2008). Alterations in adipose tissue and hepatic lipid kinetics in obese men and women with nonalcoholic fatty liver disease. *Gastroenterology*, 134(2), 424-431. doi:10.1053/j.gastro.2007.11.038.
PMC2705923.
 50. Lytle, K. A., Bush, N. C., Triay, J. M., Kellogg, T. A., Kendrick, M. L., Swain, J. M., Gathaiya, N. W., Hames, K. C., & Jensen, M. D. (2019). Hepatic fatty acid balance and hepatic fat content in humans with severe obesity. *J Clin Endocrinol Metab*, 104(12), 6171-6181. doi:10.1210/jc.2019-00875.
PMC6821207.
 51. Sondergaard, E., Rahbek, I., Sorensen, L. P., Christiansen, J. S., Gormsen, L. C., Jensen, M. D., & Nielsen, S. (2011). Effects of exercise on VLDL-triglyceride oxidation and turnover. *Am J Physiol Endocrinol Metab*, 300(5), E939-944. doi:10.1152/ajpendo.00031.2011. **PMC3279302.**
 52. Chalasani, N., Wilson, L., Kleiner, D. E., Cummings, O. W., Brunt, E. M., Unalp, A., & Network, N. C. R. (2008). Relationship of steatosis grade and zonal location to histological features of steatohepatitis in adult patients with non-alcoholic fatty liver disease. *J Hepatol*, 48(5), 829-834. doi:10.1016/j.jhep.2008.01.016. **PMC2346454.**
 53. Bril, F., Barb, D., Portillo-Sanchez, P., Biernacki, D., Lomonaco, R., Suman, A., Weber, M. H., Budd, J. T., Lupi, M. E., & Cusi, K. (2017). Metabolic and histological implications of intrahepatic triglyceride content in nonalcoholic fatty liver disease. *Hepatology*, 65(4), 1132-1144. doi:10.1002/hep.28985. **PMID: 27981615.**
 54. Shores, N. J., Fernandez, A. Z., Geisinger, K., Howerton, R., Kavanagh, K., McNatt, S., Davis, M., Nguyen, T., Sawyer, J. K., & Larry, R. (2010). *Hepatic acyl-CoA:Cholesterol O-acyltransferase 2 (Acat2) activity predicts hepatic steatosis in humans.* Paper presented at the AASLD/AGA.
 55. Lawitz, E., Li, K., Tarrant, J., Vimal, M., Xu, R., Song, Q., Aguilar Schall, R. E., McColgan, B. J., Djedjos, C. S., Ray, A. S., Myers, R. P., Hellerstein,

- M. K., & Loomba, R. (2017). *Hepatic de novo lipogenesis is elevated in patients with NASH independent of disease severity*. Paper presented at the American Association for the Study of Liver Diseases, Washington, DC.
56. Softic, S., Cohen, D. E., & Kahn, C. R. (2016). Role of dietary fructose and hepatic de novo lipogenesis in fatty liver disease. *Dig Dis Sci*, *61*(5), 1282-1293. doi:10.1007/s10620-016-4054-0. **PMC4838515**.
 57. Diraison, F., Moulin, P., & Beylot, M. (2003). Contribution of hepatic de novo lipogenesis and reesterification of plasma non esterified fatty acids to plasma triglyceride synthesis during non-alcoholic fatty liver disease. *Diabetes Metab*, *29*(5), 478-485. doi:10.1016/s1262-3636(07)70061-7. **PMID: 14631324**.
 58. Lee, J. J., Lambert, J. E., Hovhannisyan, Y., Ramos-Roman, M. A., Trombold, J. R., Wagner, D. A., & Parks, E. J. (2015). Palmitoleic acid is elevated in fatty liver disease and reflects hepatic lipogenesis. *Am J Clin Nutr*, *101*(1), 34-43. doi:10.3945/ajcn.114.092262. **PMC4266891**.
 59. Zhu, L., Baker, S. S., Liu, W., Tao, M. H., Patel, R., Nowak, N. J., & Baker, R. D. (2011). Lipid in the livers of adolescents with nonalcoholic steatohepatitis: combined effects of pathways on steatosis. *Metabolism*, *60*(7), 1001-1011. doi:10.1016/j.metabol.2010.10.003. **PMID: 21075404**.
 60. Mitsuyoshi, H., Yasui, K., Harano, Y., Endo, M., Tsuji, K., Minami, M., Itoh, Y., Okanoue, T., & Yoshikawa, T. (2009). Analysis of hepatic genes involved in the metabolism of fatty acids and iron in nonalcoholic fatty liver disease. *Hepatol Res*, *39*(4), 366-373. doi:10.1111/j.1872-034X.2008.00464.x. **PMID: 19054139**.
 61. Dorn, C., Riener, M. O., Kirovski, G., Saugspier, M., Steib, K., Weiss, T. S., Gabele, E., Kristiansen, G., Hartmann, A., & Hellerbrand, C. (2010). Expression of fatty acid synthase in nonalcoholic fatty liver disease. *Int J Clin Exp Pathol*, *3*(5), 505-514. **PMC2897101**.
 62. Araya, J., Rodrigo, R., Pettinelli, P., Araya, A. V., Poniachik, J., & Videla, L. A. (2010). Decreased liver fatty acid delta-6 and delta-5 desaturase activity in obese patients. *Obesity (Silver Spring)*, *18*(7), 1460-1463. doi:10.1038/oby.2009.379. **PMID: 1987598**.
 63. Chen, Z., Tian, R., She, Z., Cai, J., & Li, H. (2020). Role of oxidative stress in the pathogenesis of nonalcoholic fatty liver disease. *Free Radic Biol Med*, *152*, 116-141. doi:10.1016/j.freeradbiomed.2020.02.025. **PMID: 32156524**.
 64. Koliaki, C., Szendroedi, J., Kaul, K., Jelenik, T., Nowotny, P., Jankowiak, F., Herder, C., Carstensen, M., Krausch, M., Knoefel, W. T., Schlensak, M., & Roden, M. (2015). Adaptation of hepatic mitochondrial function in humans with non-alcoholic fatty liver is lost in steatohepatitis. *Cell Metab*, *21*(5), 739-746. doi:10.1016/j.cmet.2015.04.004. **PMID: 25955209**.
 65. Czech, M. P., Tencerova, M., Pedersen, D. J., & Aouadi, M. (2013). Insulin signalling mechanisms for triacylglycerol storage. *Diabetologia*, *56*(5), 949-964. doi:10.1007/s00125-013-2869-1. **PMC3652374**.

66. Wurie, H. R., Buckett, L., & Zammit, V. A. (2012). Diacylglycerol acyltransferase 2 acts upstream of diacylglycerol acyltransferase 1 and utilizes nascent diglycerides and de novo synthesized fatty acids in HepG2 cells. *FEBS J*, *279*(17), 3033-3047. doi:10.1111/j.1742-4658.2012.08684.x. **PMID: 22748069**.
67. Yen, C. L., Stone, S. J., Koliwad, S., Harris, C., & Farese, R. V., Jr. (2008). Thematic review series: glycerolipids. DGAT enzymes and triacylglycerol biosynthesis. *J Lipid Res*, *49*(11), 2283-2301. doi:10.1194/jlr.R800018-JLR200. **PMC3837458**.
68. Alves-Bezerra, M., & Cohen, D. E. (2017). Triglyceride metabolism in the liver. *Compr Physiol*, *8*(1), 1-8. doi:10.1002/cphy.c170012. **PMC6376873**.
69. Zammit, V. A. (2013). Hepatic triacylglycerol synthesis and secretion: DGAT2 as the link between glycaemia and triglyceridaemia. *Biochem J*, *451*(1), 1-12. doi:10.1042/BJ20121689. **PMID: 23489367**.
70. Meegalla, R. L., Billheimer, J. T., & Cheng, D. (2002). Concerted elevation of acyl-coenzyme A:diacylglycerol acyltransferase (DGAT) activity through independent stimulation of mRNA expression of DGAT1 and DGAT2 by carbohydrate and insulin. *Biochem Biophys Res Commun*, *298*(3), 317-323. doi:10.1016/s0006-291x(02)02466-x. **PMID: 12413942**.
71. Shin, E., Bae, J. S., Han, J. Y., Lee, J., Jeong, Y. S., Lee, H. J., Ahn, Y. H., & Cha, J. Y. (2016). Hepatic DGAT2 gene expression is regulated by the synergistic action of ChREBP and SP1 in HepG2 cells. *Animal Cells and Systems*, *20*(1), 7-14. doi:10.1080/19768354.2015.1131738. **WOS:000371742900002**.
72. Jung, S., Choi, M., Choi, K., Kwon, E. B., Kang, M., Kim, D. E., Jeong, H., Kim, J., Kim, J. H., Kim, M. O., Han, S. B., & Cho, S. (2017). Inactivation of human DGAT2 by oxidative stress on cysteine residues. *PLoS One*, *12*(7), e0181076. doi:10.1371/journal.pone.0181076. **PMC5507451**.
73. Liu, Y., Beyer, A., & Aebersold, R. (2016). On the dependency of cellular protein levels on mRNA abundance. *Cell*, *165*(3), 535-550. doi:10.1016/j.cell.2016.03.014. **PMID: 27104977**.
74. Auguet, T., Berlanga, A., Guiu-Jurado, E., Martinez, S., Porras, J. A., Aragones, G., Sabench, F., Hernandez, M., Aguilar, C., Sirvent, J. J., Del Castillo, D., & Richart, C. (2014). Altered fatty acid metabolism-related gene expression in liver from morbidly obese women with non-alcoholic fatty liver disease. *Int J Mol Sci*, *15*(12), 22173-22187. doi:10.3390/ijms151222173. **PMC4284701**.
75. Bechmann, L. P., Gieseler, R. K., Sowa, J. P., Kahraman, A., Erhard, J., Wedemeyer, I., Emons, B., Jochum, C., Feldkamp, T., Gerken, G., & Canbay, A. (2010). Apoptosis is associated with CD36/fatty acid translocase upregulation in non-alcoholic steatohepatitis. *Liver Int*, *30*(6), 850-859. doi:10.1111/j.1478-3231.2010.02248.x. **PMID: 20408954**.
76. Rada, P., Gonzalez-Rodriguez, A., Garcia-Monzon, C., & Valverde, A. M. (2020). Understanding lipotoxicity in NAFLD pathogenesis: is CD36 a key

- driver? *Cell Death Dis*, 11(9), 802. doi:10.1038/s41419-020-03003-w. **PMC7519685**.
77. Pardina, E., Ferrer, R., Rossell, J., Ricart-Jane, D., Mendez-Lara, K. A., Baena-Fustegueras, J. A., Lecube, A., Julve, J., & Peinado-Onsurbe, J. (2017). Hepatic CD36 downregulation parallels steatosis improvement in morbidly obese undergoing bariatric surgery. *Int J Obes (Lond)*, 41(9), 1388-1393. doi:10.1038/ijo.2017.115. **PMID: 28555086**.
78. Smith, B. K., Perry, C. G., Koves, T. R., Wright, D. C., Smith, J. C., Neuffer, P. D., Muoio, D. M., & Holloway, G. P. (2012). Identification of a novel malonyl-CoA IC(50) for CPT-I: implications for predicting in vivo fatty acid oxidation rates. *Biochem J*, 448(1), 13-20. doi:10.1042/BJ20121110. **PMC3863641**.
79. Foster, D. W. (2012). Malonyl-CoA: the regulator of fatty acid synthesis and oxidation. *J Clin Invest*, 122(6), 1958-1959. doi:10.1172/jci63967. **PMC3366419**.
80. Parks, E. J., Krauss, R. M., Christiansen, M. P., Neese, R. A., & Hellerstein, M. K. (1999). Effects of a low-fat, high-carbohydrate diet on VLDL-triglyceride assembly, production, and clearance. *J Clin Invest*, 104(8), 1087-1096. doi:10.1172/JCI6572. **PMC408572**.
81. Santoro, N., Caprio, S., Pierpont, B., Van Name, M., Savoye, M., & Parks, E. J. (2015). Hepatic de novo lipogenesis in obese youth is modulated by a common variant in the GCKR gene. *J Clin Endocrinol Metab*, 100(8), E1125-1132. doi:10.1210/jc.2015-1587. **PMC4524990**.
82. Hellerstein, M. K., Christiansen, M., Kaempfer, S., Kletke, C., Wu, K., Reid, J. S., Mulligan, K., Hellerstein, N. S., & Shackleton, C. H. (1991). Measurement of de novo hepatic lipogenesis in humans using stable isotopes. *J Clin Invest*, 87(5), 1841-1852. doi:10.1172/JCI115206. **PMC295308**.
83. Lambert, J. E., Ryan, E. A., Thomson, A. B., & Clandinin, M. T. (2013). De novo lipogenesis and cholesterol synthesis in humans with long-standing type 1 diabetes are comparable to non-diabetic individuals. *PLoS One*, 8(12), e82530. doi:10.1371/journal.pone.0082530. **PMC3871159**.
84. Heil, C. S., Wehrheim, S. S., Paithankar, K. S., & Grninger, M. (2019). Fatty acid biosynthesis: Chain-length regulation and control. *Chembiochem*, 20(18), 2298-2321. doi:10.1002/cbic.201800809. **PMID: 30908841**.
85. Abdinejad, A., Fisher, A. M., & Kumar, S. (1981). Production and utilization of butyryl-CoA by fatty acid synthetase from mammalian tissues. *Arch Biochem Biophys*, 208(1), 135-145. doi:10.1016/0003-9861(81)90132-6. **PMID: 7259173**.
86. Chao, H. W., Chao, S. W., Lin, H., Ku, H. C., & Cheng, C. F. (2019). Homeostasis of glucose and lipid in non-alcoholic fatty liver disease. *Int J Mol Sci*, 20(2). doi:10.3390/ijms20020298. **PMC6359196**.
87. Krycer, J. R., Sharpe, L. J., Luu, W., & Brown, A. J. (2010). The Akt-SREBP nexus: cell signaling meets lipid metabolism. *Trends Endocrinol Metab*, 21(5), 268-276. doi:10.1016/j.tem.2010.01.001. **PMID: 20117946**.

88. Laplante, M., & Sabatini, D. M. (2010). mTORC1 activates SREBP-1c and uncouples lipogenesis from gluconeogenesis. *Proc Natl Acad Sci U S A*, *107*(8), 3281-3282. doi:10.1073/pnas.1000323107. **PMC2840435**.
89. Han, X., Liu, C., Xue, Y., Wang, J., Xue, C., Yanagita, T., Gao, X., & Wang, Y. (2016). Long-term fatty liver-induced insulin resistance in orotic acid-induced nonalcoholic fatty liver rats. *Biosci Biotechnol Biochem*, *80*(4), 735-743. doi:10.1080/09168451.2015.1123608. **PMID: 26775542**.
90. Sunny, N. E., Parks, E. J., Browning, J. D., & Burgess, S. C. (2011). Excessive hepatic mitochondrial TCA cycle and gluconeogenesis in humans with nonalcoholic fatty liver disease. *Cell Metab*, *14*(6), 804-810. doi:10.1016/j.cmet.2011.11.004. **PMC3658280**.
91. Sharabi, K., Tavares, C. D., Rines, A. K., & Puigserver, P. (2015). Molecular pathophysiology of hepatic glucose production. *Mol Aspects Med*, *46*, 21-33. doi:10.1016/j.mam.2015.09.003. **PMC4674831**.
92. Chiappini, F., Coilly, A., Kadar, H., Gual, P., Tran, A., Desterke, C., Samuel, D., Duclos-Vallee, J. C., Touboul, D., Bertrand-Michel, J., Brunelle, A., Guettier, C., & Le Naour, F. (2017). Metabolism dysregulation induces a specific lipid signature of nonalcoholic steatohepatitis in patients. *Sci Rep*, *7*, 46658. doi:10.1038/srep46658. **PMC5402394**.
93. Yan, J. H., Guan, B. J., Gao, H. Y., & Peng, X. E. (2018). Omega-3 polyunsaturated fatty acid supplementation and non-alcoholic fatty liver disease: A meta-analysis of randomized controlled trials. *Medicine (Baltimore)*, *97*(37), e12271. doi:10.1097/MD.0000000000012271. **PMC6155966**.
94. Nakamura, M. T., Cho, H. P., & Clarke, S. D. (2000). Regulation of hepatic delta-6 desaturase expression and its role in the polyunsaturated fatty acid inhibition of fatty acid synthase gene expression in mice. *J Nutr*, *130*(6), 1561-1565. doi:10.1093/jn/130.6.1561. **PMID: 10827210**.
95. Clarke, S. D. (2001). Nonalcoholic steatosis and steatohepatitis. I. Molecular mechanism for polyunsaturated fatty acid regulation of gene transcription. *Am J Physiol Gastrointest Liver Physiol*, *281*(4), G865-869. doi:10.1152/ajpgi.2001.281.4.G865. **PMID: 11557505**.
96. Clarke, S. D. (2000). Polyunsaturated fatty acid regulation of gene transcription: a mechanism to improve energy balance and insulin resistance. *Br J Nutr*, *83 Suppl 1*, S59-66. doi:10.1017/s0007114500000969. **PMID: 10889793**.
97. Clarke, S. D. (2001). Polyunsaturated fatty acid regulation of gene transcription: a molecular mechanism to improve the metabolic syndrome. *J Nutr*, *131*(4), 1129-1132. doi:10.1093/jn/131.4.1129. **PMID: 11285313**.
98. Wilson, M. D., Blake, W. L., Salati, L. M., & Clarke, S. D. (1990). Potency of polyunsaturated and saturated fats as short-term inhibitors of hepatic lipogenesis in rats. *J Nutr*, *120*(6), 544-552. doi:10.1093/jn/120.6.544. **PMID: 2352029**.
99. Power, G. W., & Newsholme, E. A. (1997). Dietary fatty acids influence the activity and metabolic control of mitochondrial carnitine

- palmitoyltransferase I in rat heart and skeletal muscle. *J Nutr*, 127(11), 2142-2150. doi:10.1093/jn/127.11.2142. **PMID: 9349840.**
100. Kersten, S., Seydoux, J., Peters, J. M., Gonzalez, F. J., Desvergne, B., & Wahli, W. (1999). Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting. *J Clin Invest*, 103(11), 1489-1498. doi:10.1172/JCI6223. **PMC408372.**
 101. Araya, J., Rodrigo, R., Videla, L. A., Thielemann, L., Orellana, M., Pettinelli, P., & Poniachik, J. (2004). Increase in long-chain polyunsaturated fatty acid n - 6/n - 3 ratio in relation to hepatic steatosis in patients with non-alcoholic fatty liver disease. *Clin Sci (Lond)*, 106(6), 635-643. doi:10.1042/CS20030326. **PMID: 14720121.**
 102. Roumans, K. H. M., Lindeboom, L., Veeraiah, P., Remie, C. M. E., Phielix, E., Havekes, B., Bruls, Y. M. H., Brouwers, M., Stahlman, M., Alsema, M., Peters, H. P. F., de Mutsert, R., Staels, B., Taskinen, M. R., Boren, J., Schrauwen, P., & Schrauwen-Hinderling, V. B. (2020). Hepatic saturated fatty acid fraction is associated with de novo lipogenesis and hepatic insulin resistance. *Nat Commun*, 11(1), 1891. doi:10.1038/s41467-020-15684-0. **PMC7170906.**
 103. Knebel, B., Fahlbusch, P., Dille, M., Wahlers, N., Hartwig, S., Jacob, S., Kettel, U., Schiller, M., Herebian, D., Koellmer, C., Lehr, S., Muller-Wieland, D., & Kotzka, J. (2019). Fatty liver due to increased de novo lipogenesis: Alterations in the hepatic peroxisomal proteome. *Front Cell Dev Biol*, 7, 248. doi:10.3389/fcell.2019.00248. **PMC6823594.**
 104. Chong, M. F., Hodson, L., Bickerton, A. S., Roberts, R., Neville, M., Karpe, F., Frayn, K. N., & Fielding, B. A. (2008). Parallel activation of de novo lipogenesis and stearoyl-CoA desaturase activity after 3 d of high-carbohydrate feeding. *Am J Clin Nutr*, 87(4), 817-823. doi:10.1093/ajcn/87.4.817. **PMID: 18400702.**
 105. Rosqvist, F., McNeil, C. A., Pramfalk, C., Parry, S. A., Low, W. S., Cornfield, T., Fielding, B. A., & Hodson, L. (2019). Fasting hepatic de novo lipogenesis is not reliably assessed using circulating fatty acid markers. *Am J Clin Nutr*, 109(2), 260-268. doi:10.1093/ajcn/nqy304. **PMC6367991.**
 106. Garrido-Sanchez, L., Vendrell, J., Fernandez-Garcia, D., Ceperuelo-Mallafre, V., Chacon, M. R., Ocana-Wilhelmi, L., Alcaide, J., Tinahones, F. J., & Garcia-Fuentes, E. (2012). De novo lipogenesis in adipose tissue is associated with course of morbid obesity after bariatric surgery. *PLoS One*, 7(2), e31280. doi:10.1371/journal.pone.0031280. **PMC3285616.**
 107. Yoshino, M., Kayser, B. D., Yoshino, J., Stein, R. I., Reeds, D., Eagon, J. C., Eckhouse, S. R., Watrous, J. D., Jain, M., Knight, R., Schechtman, K., Patterson, B. W., & Klein, S. (2020). Effects of diet versus gastric bypass on metabolic function in diabetes. *N Engl J Med*, 383(8), 721-732. doi:10.1056/NEJMoa2003697. **PMC7456610.**
 108. Wree, A., Broderick, L., Canbay, A., Hoffman, H. M., & Feldstein, A. E. (2013). From NAFLD to NASH to cirrhosis-new insights into disease

mechanisms. *Nat Rev Gastroenterol Hepatol*, 10(11), 627-636.
doi:10.1038/nrgastro.2013.149. **PMID: 23958599.**

EXTENDED METHODOLOGY

Patient recruitment

A total of 142 patients undergoing bariatric surgery were screened to identify 127 patients who were introduced to the study during their pre-surgery orientation at the University of Missouri Bariatric Clinic. Of these, 71 patients were excluded due to scheduling issues, declined to participate, too healthy to be in the study, or were undergoing a correction/revision surgery. A total of 56 patients signed the consent form but three patients' surgery was canceled, two patients did not drink deuterated water (d₂O), one patient dropped out due to anxiety, and one patient did not respond after consenting. A total of 49 patients completed the study and their data were used for analyses.

Patient selection strategy

Based on power calculations (described later) the goal was to include at least eight patients in each group (mild, moderate, and severe). Although ~80% of bariatric surgery patients are diagnosed with some form of NAFLD/NASH, these patients are less likely to have a severe form of liver disease (1). Therefore, patients with normal lipid profiles and blood biochemistries were excluded after consenting enough patients in the mild and moderate disease groups.

The goal of the study was to recruit at least eight patients in each group, therefore, initially, all patients who fit inclusion criteria were introduced to the study and were consented. Once, enough patients were obtained in the mild and the moderate group, only patients with the characteristics listed in **extended**

table 2.1 and extended figure 2.2a-b were consented to increase the probability of including patients with severe liver disease.

Biochemical measurements

Plasma concentrations of total cholesterol, TAG, low-density lipoprotein cholesterol (LDLc), high-density lipoprotein cholesterol (HDLc), aspartate aminotransferases (AST), and alanine aminotransferases (ALT) were measured by a CLIA-standardized laboratory (#26D0652092, Quest Diagnostics, St. Louis, MO). The measurements of lipids were performed via auto-analyzer (Roche Cobas 8000 System, CV 0.6-0.9%, Indianapolis, IN) using electro-chemiluminescent immunoassay. Liver enzymes were measured using UV Absorbance (Roche Cobas 8000 System, CV 0.5-3.2% for AST and 0.5-3.1% for ALT, Indianapolis, IN). Assay kits were used to measure the concentrations of plasma glucose (#439-90901, CV 6.6%, Wako, Mountain View, CA) and nonesterified fatty acids (NEFA, #991-34891, CV 6.9%, Wako, Mountain View, CA). Plasma insulin was measured using an enzyme-linked immunosorbent assay (#EZHI-14K, Human Insulin, CV 7.2%, EMD Millipore, Billerica, MA).

Western blotting

Sample processing: A 100 mg of the liver sample was weighed and was transferred into a two ml screw-top tube under liquid nitrogen. One ml of buffer solution (see below) was added per 100 mg of frozen tissue and was homogenized using Tissue Lyser (Retsch, Inc., Newton, PA) for two minutes at

20Hz. The samples were left on ice for ten minutes and were processed on Tissue Lyser for another two minutes at 20Hz to make sure that the liver tissue was completely homogenized. The homogenized tissue was centrifuged at 15,000g for 25 minutes at 4C and the supernatant (homogenate) was transferred into a 1.5 ml Eppendorf tube. From this homogenate, ten μL of homogenate was mixed with 90 μL of distilled water in a separate tube to form 1:10 dilution. Protein contents were evaluated using the bovine serum albumin (BSA) concentration assay (BCA) kit (#23225, Thermo Fisher Scientific, Rockford, IL).

Buffer solution: Stock solution (44.2 ml) containing 50 mM HEPES (#BP310-1, Fisher Scientific, Fair Lawn, NJ), 12 mM sodium pyrophosphate (#7772-88-5, Aldrich Chemicals, Milwaukee, WI), 100 mM sodium fluoride (#S6776, Sigma, St. Louis, MO), and ten mM EDTA (#BP120-500, Fisher Scientific, Fair Lawn, NJ) was mixed with 400 μL of each phosphatase inhibitors (#P0044 and P52726, St. Louis, MO) and five ml of ten percent Triton (100X-Triton, #7-X198, JT Baker Chemicals, Phillipsburg, NJ). A protease inhibitor tablet (#1187358001, Roche Diagnostics, Indianapolis, IN) was added to this solution.

BCA run: Samples were diluted in a 1:10 ratio with distilled water. Samples (10 μL) and standard (10 μL) solutions were plated in wells and 200 μL reagent (reagent A and reagent B in a ratio of 49:1, #23223 and #23224, Thermo Fisher Scientific, Rockford, IL). The plate was left on a shaker for two minutes, in the incubator at 37C for 30 minutes, and was read at 540 nanometers. The values generated were in $\mu\text{g/ml}$ of protein. The goal was to obtain $\geq 10 \mu\text{g/ml}$ of

protein. This value was used to calculate sample volume (μl) for three $\mu\text{g}/\text{ml}$ of protein.

Sample preparation, run, and analysis: Cell lysates were prepared using Triton X-100 to which Laemmli gel loading buffer (#161-0737; Bio-Rad Laboratories, Hercules, Calif., USA) was added in a ratio of 19:1. This mixture and was boiled at 100C for ten minutes to produce Western blot-ready Laemmli samples. For each sample, 20 μg of protein was added to each well on a gel. These samples were then placed on ice for ten minutes and stored at -80C freezer for protein run.

Blots were separated using a tris-glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride transfer membrane (#88518; Thermo Fisher Scientific, Rockford, Ill., USA). The membrane was then incubated overnight in a blocking solution containing (5% dry milk in Tris-buffered saline (TBS, #BP1525, Fisher Scientific, Fair Lawn, NJ) Tween 20 buffer (#B7337-500, Fisher Scientific, Fair Lawn, NJ)) followed by overnight incubation in the primary antibody (1:1,000 dilution). After incubation, the membranes were washed three times for five minutes each with the TBS-Tween 20 buffer and placed in a secondary antibody (1:5,000 dilution) for one hour followed by three five-minute washes with the TBS-Tween 20 and two five-minute washes with TBS solution.

Western blots were analyzed via densitometric analysis using ChemiDoc™ MP Imaging System (Image Laboratory Beta 3, Bio-Rad Laboratories, Hercules, CA).

Each sample was adjusted to the average intensity of all samples on the gel. The total protein was quantitated with 0.1% Amido black (#100563, MP Biomedicals, Solon, OH) solution (500 ml distilled water, 400 ml methanol, 100 ml acetic acid, and one gm Amido black) to control for the differences in protein loading and transfer. The total protein staining for each lane, quantified by laser densitometry was used to correct for any differences in protein loading or transfer of all band densities (2, 3).

Primary and secondary antibodies: The primary antibodies used were sterol regulatory element-binding protein-1 (SREBP1; #367, Rabbit, 68 kDa, Santa Cruz Biotechnology, Dallas, TX), acetyl coenzyme A carboxylase (ACC; #3662, Rabbit, 280 kDa, Cell Signaling Technology, Danvers, MA), fatty acid synthase (FASN; #3189, Rabbit, 273 kDa, Cell Signaling Technology, Danvers, MA), stearoyl-CoA desaturase-1 (SCD1, #SCD11-A, Rabbit, 37 kDa, Alpha Diagnostics, San Antonio, TX), diacylglycerol O-Acyltransferase-1 (DGAT1, #59034, Goat, 50 kDa, Abcam, Cambridge, MA), diacylglycerol O-Acyltransferase-2 (DGAT2, #59493, Goat, 50 kDa, Abcam, Cambridge, MA), Insulin-induced gene-1 protein (INSIG, #SC-25124-R, Rabbit, 35kDa, Santa Cruz Biotechnology, Dallas, TX), cluster of differentiation 36 (CD36, #9154, Rabbit, 88 kDa, Santa Cruz Biotechnology, Dallas, TX), and Apolipoprotein B (ApoB, #20737, Mouse, 549 kDa, Abcam, Cambridge, MA). Appropriate secondary antibodies used were for each protein.

Quantitative real-time PCR

Sample preparation and RNA extraction: All the samples were processed on a bench and supplies cleaned with RNase Zap™ (#AM9780, AM9782, Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania). Frozen liver tissue was weighed (20-30 mg) under liquid nitrogen and RNA was extracted from the frozen liver using a commercially available kit (#74104, Qiagen GmbH, Germany). Liver samples were mixed with lysate buffer (RLT buffer, #1015750, Qiagen GmbH, Germany) and homogenized for four minutes (two times for two minutes at 20Hz) using Tissue Lyser (Retsch, Inc., Newton, PA). The liver homogenate was centrifuged for three minutes and the supernatant was transferred into a clean Eppendorf tube. The supernatant was mixed with 50% ethanol and was filtered by centrifuging the mixture in a two ml collection tube at 8000g for 15 seconds. The filtrate was washed with provided buffers (buffer RW1) for 15 sec at 8000g followed by washing twice with buffer RPE (15 seconds then two minutes) at 8000g. Lastly, RNA was eluted by adding 2x30 µL of Molecular Grade Water (RNase-free water, #768-293, G-Biosciences, St. Louis, MO) and centrifuged for one minute at 8000g. The purity and the quality of RNA were assessed using a NanoDrop spectrometer (model ND-1000, NanoDrop, Thermo Scientific, Wilmington, DE). These samples were stored for the next step during which the complementary deoxyribonucleic acid (cDNA) library was synthesized.

cDNA preparation: The cDNA library was synthesized as per the manufacturer's

instructions (#A3800, Promega, Madison, WI). A cocktail of random primers (#C1181, Promega, Madison, WI), reaction buffer (#M289A, Promega, Madison, WI), and magnesium chloride (#M2670-100G, Sigma, St. Louis, MO) in a 1:4:2 ratio was prepared and was added to sample (seven μL for one μL sample and ten μL RNase-free water) in 0.2 ml PCR eight-strip tubes (#AVSST-C, MIDSCI, St. Louis, MO). The mixture was incubated for five minutes at 70C followed by five minutes at 4C and were then left on ice for ten minutes. Meanwhile, a cocktail of ImProm-IITM Reverse Transcriptase (#A3802, Promega, Madison, WI) and deoxynucleoside triphosphate (dNTP, #U151B, Promega, Milwaukee, WI) was prepared and two μL was added to the sample mix. The samples were centrifuged for two seconds in a mini centrifuge (#SC1006-R, Benchmark Scientific, Sayreville, NJ) and were placed on a thermocycler (VeritiTM 96-well Thermal Cycler, Applied Bioscience, Thermo Fisher Scientific, Singapore) at 25C for four minutes, followed by 42C for 40 minutes, and 70C for 15 minutes, and 4C for the remaining time, to synthesize the cDNA library. The purity and the quality of cDNA were assessed using a Nanodrop spectrometer (model ND-1000, NanoDrop, Thermo Scientific, Wilmington, DE). These samples were diluted with RNase-free water to obtain a final sample concentration of ten $\mu\text{g}/\mu\text{L}$.

Sample run and analysis: Quantitative real-time PCR (qRT-PCR) was conducted using SYBR Green reagents (#172–5121, BioRad, Hercules, CA) and primer pairs listed in **table 2.2** (Sigma). The primer pairs cocktail (forward, reverse, RNase free water in a ratio of 1:1:8) was mixed with the SYBR Green reagent

(1:10 ratio, e.g., one ml primers mix + ten ml SYBR Green reagent). The mixture (11 μ L) was placed in the qPCR plate wells (#AVT3890, MIDSCI, St. Louis, MO) to which ten μ L of the sample was added (triplicate of each sample). The plate was sealed with Avant Thermal Seal (#TS-RT2-100, MIDSCI, St. Louis, MO) and was centrifuged at 500g for five minutes at 4C. The samples were run on the 7500 Fast Real-Time PCR System (Applied Biosciences, Singapore), the PCR product melt curves were used to assess primer specificity. PCR product melt curves were used to assess primer specificity. Data are represented relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the $2^{-\Delta\Delta CT}$ method (2-4).

Liver TAG content

Frozen liver samples (approximately 30 mg) were homogenized in chloroform and methanol (2:1) mixture using Tissue Lyser for two minutes at 20Hz. The homogenization process was repeated if the tissue was not completely homogenized during the first two minutes. The liver homogenate was left overnight on a Labquake Rotisserie Shaker (#4152110, Thermo Fisher Scientific, Waltham, MA) for the complete isolation of lipids from the tissue through gentle agitation. The next day, magnesium chloride (4mM) was added to the homogenate, vortexed, and centrifuged for one hour at 1000g at 4C. The organic phase (500 μ L of the bottom layer) was drawn carefully and was transferred in a new tube and the samples were left overnight for the evaporation of the organic solvent. On day three, samples were reconstituted in 500 μ L tert-

butanol (#47172, Sigma, St. Louis, MO) and triton-x114 mix (#X114, Sigma, St. Louis) mixed in a ratio of 3:2, respectively. Standards were prepared using Glycerol Standard (#G7793, Sigma, St. Louis, MO) reagent with serial dilutions using RNase-free water to form the concentrations 0.125, 0.625, 0.312, 0.156, 0.078 $\mu\text{Mol/L}$. Both the standards and samples (3 μL) were placed in wells in triplicates to which 300 μL of the working reagent (Triglyceride Reagent, #T2449, and Free Glycerol Reagent, #F6428, Sigma, St. Louis, MO) was added. Samples were incubated for 40 minutes at 37C and were run at 540 nanometers on an Absorbance Reader (Elx808, BioTek, Winooski, VT). The final values were reported in mg/g of tissue wet weight (2).

DNL measurements in the liver: Liver samples (100mg) were homogenized using Bio-Gen homogenizer (#PRO200, PRO Scientific Inc., Oxford, CT) in six ml folch solution (chloroform and methanol in 2:1 ratio) containing internal standards for TAG-19:0 (#T4632-1G, Sigma, St. Louis, MO), CE-17:0 (#CH-816-S5-C, Nu-Chek Prep, Inc., Elysian, MN), and FFA-15:0 (#P6125-5G, Sigma, St. Louis, MO) in a proportion as expected in normal and disease liver (5). Total lipids were extracted using the method described previously (6). The liver-TAG, liver-CE, and liver-FFA were separated via thin-layer chromatography and FAME prepared as described previously (7). Labeled FAMES were measured using a 6890N gas chromatography coupled to a 5975 mass spectrophotometry detector (Agilent Technologies, Palo Alto, CA) using a DB-225 column (20m length, inner diameter 0.180mm, and 0.20 μm film, Part# 121-2223, Agilent J&W GC Columns, Chrom

Tech, Inc., Apple Valley, MN) and helium as a carrier gas. The electron impact was used to selectively monitor ions with mass/charge (m/z) ratios of M0, M1, and M2. Targeted m/z ratios were 242.4, 243.4, and 244.4 for 14:0, 270.5, 271.5, and 272.5 for 16:0 and 298.5, 299.5, and 300.5 for 18:0. Targeted ion peak area of 200,000 for M0 in both the standard (Supelco 37 Component FAME Mix, FAME37, EC# 200-838-9, Sigma, St. Louis, MO) and samples were achieved by either diluting or concentrating the sample. In some cases, the amount of volume injected was adjusted to achieve the targeted ion peak area of 200,000. The percent DNL for 14:0, 16:0, and 18:0 were calculated by mass isotopomer distribution analysis (8). Unless specified, the DNL data presented are from 16:0 FA. Using the conventional method, absolute DNL was calculated by multiplying the percent DNL in liver-TAG, liver-CE, and liver-FFA by the total liver-TAG, liver-CE, and liver-FFA content, respectively (9). Therefore, the absolute DNL was represented as mg/g of tissue wet weight.

A separate analysis was also performed in which the percent DNL of each FA (14:0, 16:0, and 18:0) was multiplied by their respective concentrations (instead of total concentrations). The final value obtained represented the absolute DNL in the specific FA (14:0, 16:0, or 18:0). The sum absolute DNL from these three FAs (14:0, 16:0, and 18:0) were collectively represented as total absolute DNL for each lipid class. Calculations were made for liver-TAG, liver-CE, and liver-FFA absolute DNL. The sum of absolute DNL from all three lipid classes was reported as total absolute DNL in the liver.

DNL measurements in the TRL particle: Plasma samples were processed to isolate TRL using ultracentrifugation at 40,000 rpm at 15°C in a 50.3Ti rotor (Beckman Instruments, Palo Alto, CA) for 20h (7). The TRL fraction (2 ml from the upper layer) was collected by tube slicing. Total lipids were extracted using the method described previously (6). The TRL-TAG and TRL-CE were separated via thin-layer chromatography and FAME were measured using a 6890N gas chromatography coupled to a 5975 mass spectrophotometry detector (Agilent Technologies, Palo Alto, CA) as described above. The percent DNL for 14:0, 16:0, and 18:0 were calculated by mass isotopomer distribution analysis (8, 10). Absolute DNL was calculated by multiplying the percent DNL in TRL-TAG by the concentration of TRL-TAG (9). Therefore, the absolute DNL was represented as mg/dL.

DNL calculations: DNL was also calculated using the following formula presented in Bederman et al (11):

$$\text{DNL}\% = \frac{\text{total \% } 2\text{H labeling palmitate}}{\text{2H labeling body water} \times \text{N}} \times 100$$

Where 2H is ²H - deuterated water, total % 2H labeling is excess M1, 2H labeling body water is deuterium enrichment, and N is the number of deuterium that can replace hydrogens in a FA. Based on the experiments conducted by Patton et al (12), the following N was used for each FA. Myristate (14:0): N=17, palmitate (16:0): N=19.5, and stearate (18:0): N=22.

FA composition: FA composition of liver-TAG, liver-CE, liver-FFA, TRL-TAG, and TRL-CE was quantitated using FAME were measured using a 7890B gas chromatography (Agilent Technologies, Palo Alto, CA) using a DB-23 column (60m length, inner diameter 0.250mm, 0.15 μ m film, and seven-inch cage, Part# 122-2361, Agilent J&W GC Columns, Chrom Tech, Inc., Apple Valley, MN) and hydrogen as a carrier gas, helium as a makeup gas, and air. Individual FAMES were identified using their retention time and the areas were collected for individual FAME in Microsoft excel. The percent of each FA was calculated by dividing the area of each FA by the sum of total FA. For liver-TAG and TRL-TAG FA composition, percent areas were multiplied by total TAG concentration (obtained through enzymatic assay) to obtain each FA concentration in mg/g of tissue (for liver-TAG) and mg/dL (for TRL-TAG). For liver-CE and liver-FFA, known concentrations of internal standards (17:0 for CE and 15:0 for FFA) were used to calculate the concentration of each FA and total CE and FFA concentrations in the liver.

Calculations

Liver weight was calculated by using the formula as described by Chan et al (13):
Liver weight (grams) = [(218 + BW (kg) * 12.3) + (gender*51)] where male = 1, female = 0.

Power calculations: Based on previous literature (14-18), mean values of fasting DNL for healthy (<10%), obese (14-15%), and NAFLD patients with high liver fat

(>20%) were at least five percent different. Based on these data, patients in the highest tertile (NAS 5-8) were hypothesized to have DNL values at least five percent greater than the patients in the medium tertile (NAS 3-5) and patients in the medium tertile were hypothesized to have DNL value five percent greater than the patients in the low tertile (NAS 0-2). With 90% power to detect a statistical-significance and clinically-meaningful differences in DNL of five percent with a standard deviation of 3%, at least eight subjects were needed per group.

Insulin resistance calculators: The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated as $[(\text{glucose in mg/dL} \times \text{insulin in } \mu\text{U/mL}) / 405]$ (19), the quantitative insulin sensitivity check index (QUICKI) as $[1 / (\log(\text{insulin in } \mu\text{U/mL}) + \log(\text{glucose in mg/dL}))]$ (20), and adipose tissue insulin resistance (AdipoIR) was calculated as $[(\text{insulin in } \mu\text{U/L} \times \text{NEFA in mmol/L}) \times 6.9444]$ (21).

NAFLD calculators: The NAFLD fibrosis score (FS) was calculated using the formula (22): $\text{FS} = -1.675 + [0.037 \times \text{age (years)}] + [0.094 \times \text{BMI (kg/m}^2)] + [1.13 \times \text{hyperglycemia or diabetes (yes = 1, no = 0)}] + [0.99 \times \text{AST/ALT ratio}] - [0.013 \times \text{platelet (x10}^9 \text{/L)}] - [0.66 \times \text{albumin (g/dL)}]$, and fibrosis index based on the 4-factors (FIB-4) was calculated using the formula (23): $\text{FIB-4} = [\text{age (years)} \times \text{AST (U/L)}] / [\text{platelet (10}^9 \text{/L)} \times \sqrt{\text{ALT (U/L)}}]$. The Hepatic Steatosis Index (HSI) was calculated using the formula (24): $\text{HSI} = 8 \times \text{ALT/AST ratio} + \text{BMI} + 2 \text{ (if DM)} + 2 \text{ (if female)}$, the NAFLD Liver Fat Score (LFS) was calculated using the formula

(25): $LFS = -2.89 + 1.18 \times \text{metabolic syndrome (yes = 1/ no = 0)} + 0.45 \times \text{type 2 diabetes (yes = 2/ no = 0)} + 0.15 \times \text{fasting insulin (U/L)} + 0.04 \times \text{fasting AST (U/L)} - 0.94 \times \text{AST/ALT}$, Dallas Steatosis Index (DSI) was calculated using the formula

(26): $DSI = -9.388 + 0.316 \text{ (if } \geq 50 \text{ years of age and female)} + 2.43 \text{ (if known diabetes)} + 0.019 \times \text{(equals 0 if diabetic; if not diabetic equals the glucose concentration in mg/dL)} + 0.288 \text{ (if known hypertension)} + 0.495 \text{ (if Hispanic/Asian/Other race/ethnicity)} + \text{Ln(triglycerides in mg/dL)} + 0.408 \text{ (if alanine aminotransferase [ALT] 13.5 - 19.49 IU/L)} + 1.107 \text{ (if ALT 19.5 - 40 IU/L)} + 1.515 \text{ (if ALT } > 40 \text{ IU/L)} + 0.692 \text{ (if not black and BMI 25-27.49 kg/m}^2\text{)} + 1.429 \text{ (if not black and BMI 27.5 - 34.9 kg/m}^2\text{)} + 1.933 \text{ (if not black and 35 - 37.49 kg/m}^2\text{)} + 2.643 \text{ (if not black and } > 37.5 \text{ kg/m}^2\text{)} - 0.163 \text{ (if black and BMI 25 - 27.49 kg/m}^2\text{)} + 0.882 \text{ (if black and BMI 27.5 - 34.9 kg/m}^2\text{)} + 0.759 \text{ (if black and 35 - 37.49 kg/m}^2\text{)} + 1.806 \text{ (if black and } > 37.5 \text{ kg/m}^2\text{)}$, the predicted DSI NAFLD risk was calculated using the formula (26): $e^{DSI} / (1 + e^{DSI})$, Framingham Steatosis Index (FSI) was calculated using the formula (27): $FSI = -7.981 + 0.011 \times \text{age (years)} - 0.146 \times \text{sex (female = 1, male = 0)} + 0.173 \times \text{BMI (kg/m}^2\text{)} + 0.007 \times \text{triglycerides (mg/dL)} + 0.593 \times \text{hypertension (yes = 1, no = 0)} + 0.789 \times \text{diabetes (yes = 1, no = 0)} + 1.1 \times \text{ALT/AST ratio } \geq 1.33 \text{ (yes = 1, no = 0)}$, BARD score (**B**MI, **A**ST/ALT ratio, **d**iabetes) was calculated using the formula (28) $BARD = 1 \text{ point (if BMI } > 28) + 2 \text{ points if AST/ALT ratio } > 0.8 + 1 \text{ point (if diabetic)}$, the aspartate aminotransferase to platelet ratio index (APRI) score was calculated using the formula (29): $APRI = (\text{AST (U/L) / upper limit normal}) \times 100 / \text{platelet } 10^9/\text{L}$, and FibroScan-AST (FAST) score was calculation using the formula (30):

$$\text{FAST} = (\text{EXP}(-1.65+1.07*\text{LN}(\text{LSM})+2.66*10^{-8}*(\text{CAP})^3-63.3*(\text{AST U/L})^{-1}))/((1+\text{EXP}(-1.65+1.07*\text{LN}(\text{LSM})+2.66*10^{-8}*(\text{CAP})^3-63.3*(\text{AST})^{-1}))).$$

Lipid subclass calculations: Total FA in each lipid class (TAG, CE, and FFA) was calculated by adding the concentration of each FA (measured by GC). For subclass analysis, saturated FA (SFA), monounsaturated FA (MUFA), polyunsaturated FA (PUFA) were calculated. Additionally, separate calculations were performed to calculate total omega-3 FAs, omega-6 FAs, eicosapentaenoic acid and docosahexaenoic acid (EPA+DHA), essential FAs, and non-essential FAs. The SFA concentration was calculated by adding the concentrations of 14:0, 16:0, and 18:0 FA, similarly, MUFA included the sum of 16:1n7, 18:1n9, and 18:1n7 FA, PUFA included the sum of 18:2n6, 18:3n6, 18:3n3, 20:3n6, 20:4n6, 20:5n3, 22:4n6, 22:5n6, 22:5n3, and 22:6n3 FA, omega-3 FAs included the sum of 18:3n3, 20:5n3, 22:5n3, and 22:6n3 FA, omega-6 FAs included the sum of 18:2n6, 18:3n6, 20:3n6, 20:4n6, 22:4n6, and 22:5n6 FA, essential FAs included the sum of 18:2n6, 18:3n6, 18:3n3, 20:3n6, 20:4n6, 20:5n3, 22:4n6, 22:5n6, 22:5n3, and 22:6n3 FA, and non-essential FAs included the sum of 14:0, 16:0, 16:1n7, 18:0, 18:1n9, and 18:1n7 FA. The percent of each subclass was calculated by dividing the subclass concentration by the total concentration. For SFA, MUFA, and PUFA, the sum of each subclass was divided by the sum of all FA. For omega-3 FAs, omega-6 FAs, and EPA+DHA, the percent of each subclass was reported for percent of total FA but not percent of PUFA.

DNL indices calculations: DNL indices were calculated using the FA composition data collected via GC in liver-TAG, liver-CE, liver-FFA, TRL-TAG, and TRL-CE. The LD was calculated by using the area ratio of 16:0/18:2 (31), SCD₍₁₆₎ index was calculated by using the area ratio of 16:1n7/16:0 (32), SCD₍₁₈₎ index was also calculated by using the area ratio of 18:1n9/18:0 (33, 34), and elongation indices were calculated by using the area ratio of 18:0/16:0 and 18:1n7/16:1n7 (33, 35).

EXTENDED RESULTS

For the reader's interest, shown in **extended table 2.2** are the percent and absolute DNL calculated from both methods (Hellerstein et al and Bederman et al). The agreement between both methods was significantly strong (**extended figure 2.3**). the data presented compared the percent DNL in liver TAG 16:0. Similar results were obtained for 14:0 and 18:0 in liver-CE, liver-FFA, TRL-TAG, and TRL-CE (data not shown).

Extended Table 2.1: Patient selection strategy

Tools	Strategy
High AST, plasma TG, BMI, GGT, insulin, low HDL, high HOMA-IR score	Patients with these characteristics were likely to have a severe form of NAFLD.
High HbA1c	Diabetic compared to nondiabetic individuals are more likely to have NAFLD, therefore, HbA1c was also screened.
Ethnicity	African Americans are protected from NAFLD.
Prior diagnosis	Patients already diagnosed with NAFLD were included after talking to them about the severity of the disease.
Patient's motivation	Patients motivated to participate are less likely to drop off and follow instructions.

Legend: Seventeen studies in NAFLD patients were reviewed to identify factors that would predict the presence of NAFLD. Nineteen variables identified as predictors of NAFLD were as follows: Alanine transaminase (ALT), plasma triglycerides (TG), body mass index (BMI), gamma-glutamyl transferase (GGT), plasma insulin, homeostasis model for insulin resistance (HOMA-IR), aspartate aminotransferases (AST), high-density lipoprotein cholesterol (HDLc), uric acid (UA), hypertension status (HTN), plasma glucose, ferritin, diabetic status, glycohemoglobin A1C (HbA1c), total cholesterol (TC), low-density lipoprotein cholesterol (LDLc), waist to hip ratio (W:H), alkaline phosphatase (ALP), and c-peptide. Of these top eight listed were selected to screen patients before surgery.

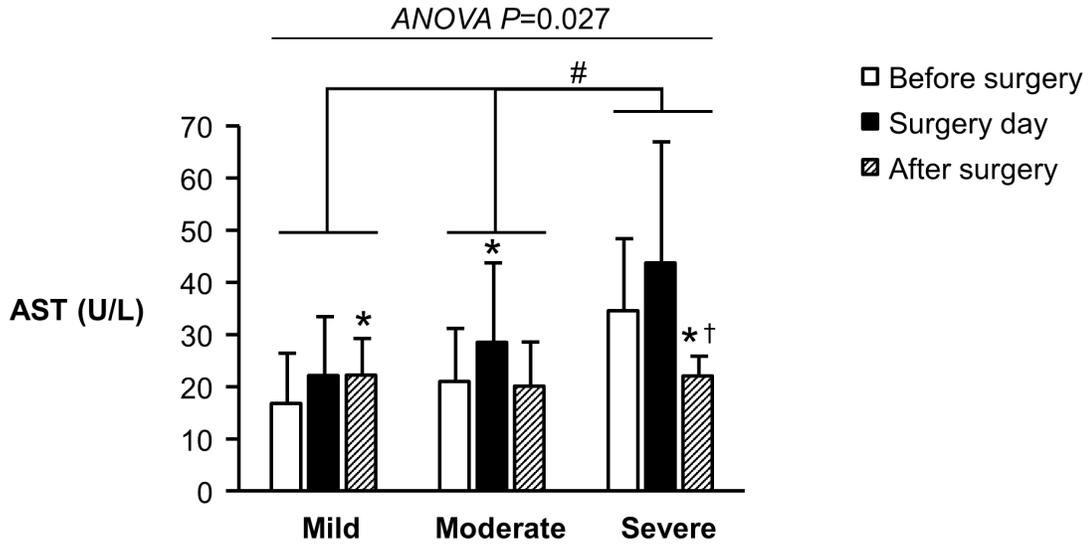
Extended Table 2.2: DNL data calculated using both methods

Bederman et al		Liver-TG-14:0	Liver-TG-16:0	Liver-TG-18:0	Liver-CE-14:0	Liver-CE-16:0	Liver-CE-18:0	Liver-FFA-14:0	Liver-FFA-16:0	Liver-FFA-18:0	TRL-TG-14:0	TRL-TG-16:0	TRL-TG-18:0	TRL-CE-14:0	TRL-CE-16:0	TRL-CE-18:0
<i>Percent DNL</i>																
Mild	<i>Mean</i>	23%	4%	9%	17%	3%	11%	15%	6%	9%	20%	5%	13%	18%	5%	11%
	<i>SD</i>	12%	2%	5%	10%	2%	10%	6%	3%	4%	8%	3%	5%	7%	4%	4%
Moderate	<i>Mean</i>	24%	10%	14%	22%	6%	13%	14%	5%	10%	22%	8%	12%	15%	6%	11%
	<i>SD</i>	16%	5%	7%	11%	4%	8%	6%	2%	5%	9%	4%	6%	6%	4%	7%
Severe	<i>Mean</i>	15%	3%	11%	18%	5%	9%	12%	5%	11%	17%	5%	13%	15%	6%	11%
	<i>SD</i>	7%	1%	7%	15%	2%	5%	8%	2%	4%	6%	3%	7%	6%	2%	6%
<i>Absolute DNL (mg/dL)</i>																
Mild	<i>Mean</i>	2.4	0.5	1.0	3.0	0.7	2.0	0.4	0.2	0.3	15.5	4.3	10.9	–	–	–
	<i>SD</i>	2.1	0.4	0.9	2.2	0.6	1.7	0.3	0.1	0.2	11.0	4.6	9.2	–	–	–
Moderate	<i>Mean</i>	13.0	3.8	7.0	7.5	1.7	3.7	0.3	0.1	0.2	14.7	6.6	8.9	–	–	–
	<i>SD</i>	15.3	2.4	7.5	8.6	1.5	2.4	0.2	0.1	0.1	10.8	7.3	8.4	–	–	–
Severe	<i>Mean</i>	12.7	3.3	10.9	9.6	2.3	4.3	0.3	0.1	0.3	15.8	4.5	12.0	–	–	–
	<i>SD</i>	5.8	2.4	8.0	10.8	0.9	2.4	0.2	0.1	0.2	8.3	4.0	7.2	–	–	–
Hellerstein et al																
<i>Percent DNL</i>																
Mild	<i>Mean</i>	30%	6%	15%	22%	5%	19%	19%	8%	16%	26%	7%	22%	23%	8%	19%
	<i>SD</i>	15%	3%	9%	13%	3%	16%	8%	4%	7%	10%	4%	9%	9%	6%	6%
Moderate	<i>Mean</i>	30%	14%	24%	29%	8%	21%	17%	8%	17%	29%	12%	21%	20%	9%	19%
	<i>SD</i>	19%	6%	12%	15%	5%	13%	8%	3%	8%	11%	5%	10%	8%	5%	10%
Severe	<i>Mean</i>	20%	5%	19%	23%	8%	16%	15%	8%	18%	23%	7%	23%	19%	9%	19%
	<i>SD</i>	9%	2%	11%	19%	3%	8%	10%	3%	7%	7%	4%	12%	8%	3%	10%
<i>Absolute DNL (mg/dL)</i>																
Mild	<i>Mean</i>	3.0	0.7	1.6	3.9	1.0	3.3	0.5	0.2	0.5	19.9	6.2	18.2	–	–	–
	<i>SD</i>	2.6	0.6	1.5	2.8	0.9	2.6	0.3	0.1	0.4	13.4	6.4	15.0	–	–	–
Moderate	<i>Mean</i>	17.0	5.6	11.9	9.8	2.5	6.2	0.4	0.2	0.4	18.7	9.5	14.8	–	–	–
	<i>SD</i>	20.3	3.7	12.9	11.6	2.2	4.0	0.2	0.1	0.2	12.5	10.0	13.0	–	–	–
Severe	<i>Mean</i>	16.6	4.9	18.4	12.5	3.4	7.3	0.4	0.2	0.6	20.7	6.7	20.3	–	–	–
	<i>SD</i>	7.5	3.6	13.4	14.2	1.4	4.1	0.2	0.1	0.3	10.8	5.9	12.2	–	–	–

Legend: Data are presented in mean and SD. mild: n= 20, moderate %DNL n=20, aDNL, n=19 , severe n=9.

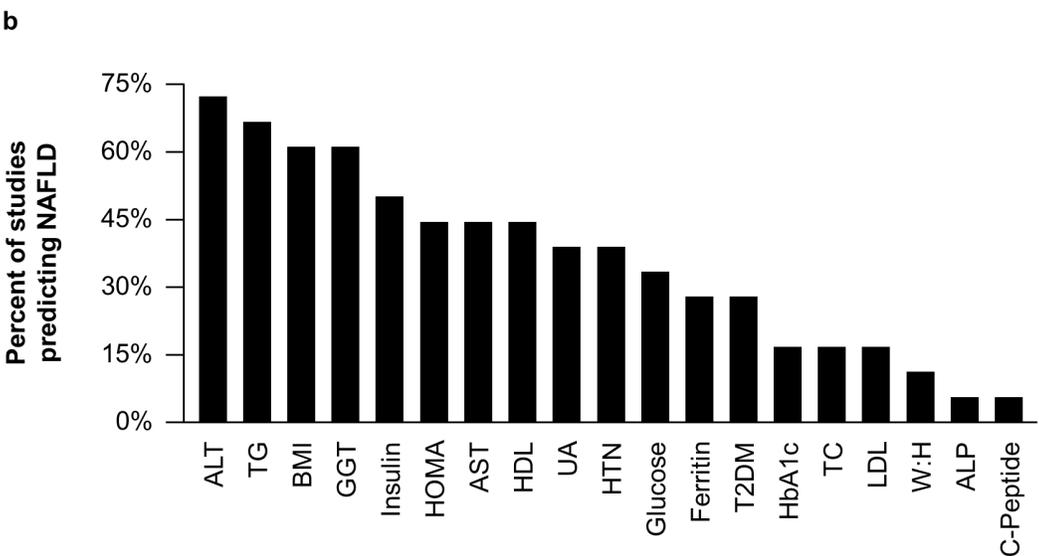
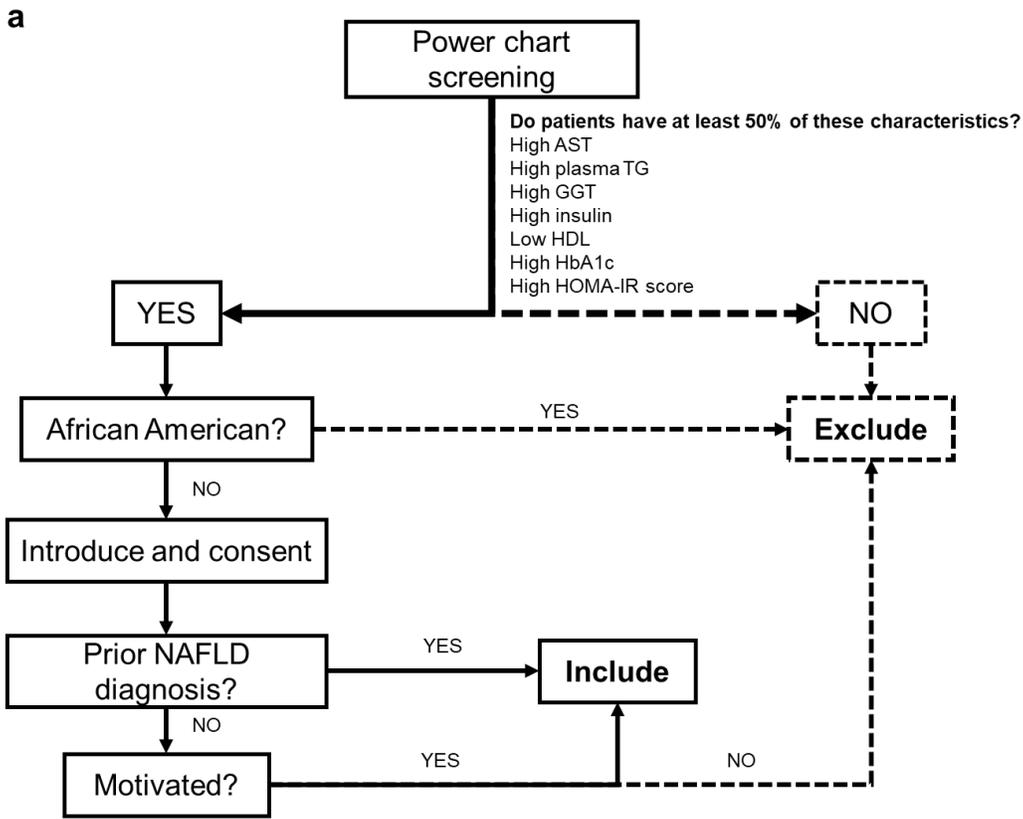
Extended Figure 2. 1: Changes in AST before and after bariatric surgery

$P < 0.05$ between group comparison
 * $P < 0.05$ compared to mild group
 † $P < 0.05$ compared to moderate group



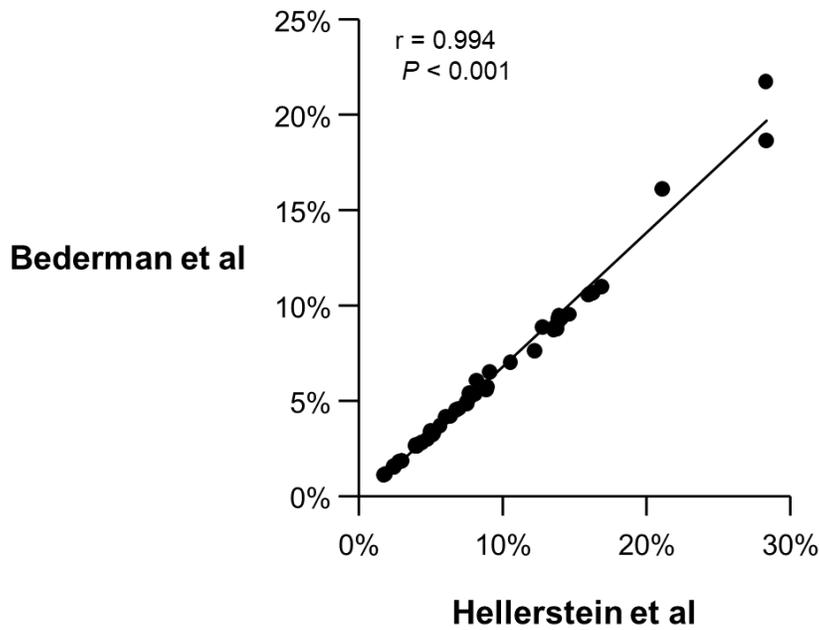
Legend: Data are reported in mean±SD. Two-factor ANOVA was conducted using time as a between-subject variable and disease severity as a within-subject variable. ANOVA P is presented above the graph. Tuckey's post-hoc analysis was performed to reveal that the severe group's AST was significantly different from the other two groups (#). Within-group comparisons revealed that the AST values were significantly higher after surgery in the mild group. In the moderate group, AST values were significantly higher but were returned to normal after surgery. Lastly, in the severe group, AST values were significantly reduced after surgery compared to the values obtained from before and on the day of surgery.

Extended Figure 2. 2: Patient selection strategy and frequency of metabolic variables used in previous studies to predict NAFLD



Legend: **a.** Flowchart of patient selection strategy. **b.** n=17 studies. Data represent the percent of studies that reported metabolic variables listed on the x-axis. For example, 72% of previous studies reported ALT as a metabolic marker for predicting NAFLD.

Extended Figure 2. 3: Agreement between two methods for calculating DNL



Legend: Data presents the percent DNL in liver-TAG 16:0. A bivariate Pearson correlation analysis was performed to test the relationship between percent DNL obtained using Hellerstein et al formula vs the formula provided in Bederman et al.

EXTENDED REFERENCES

1. Soresi, M., Cabibi, D., Giglio, R. V., Martorana, S., Guercio, G., Porcasi, R., Terranova, A., Lazzaro, L. A., Emma, M. R., Augello, G., Cervello, M., Pantuso, G., Montalto, G., & Giannitrapani, L. (2020). The prevalence of NAFLD and fibrosis in bariatric surgery patients and the reliability of noninvasive diagnostic methods. *Biomed Res Int*, 2020, 5023157. doi:10.1155/2020/5023157. **PMC7201516**.
2. Rector, R. S., Thyfault, J. P., Morris, R. T., Laye, M. J., Borengasser, S. J., Booth, F. W., & Ibdah, J. A. (2008). Daily exercise increases hepatic fatty acid oxidation and prevents steatosis in Otsuka Long-Evans Tokushima Fatty rats. *Am J Physiol Gastrointest Liver Physiol*, 294(3), G619-626. doi:10.1152/ajpgi.00428.2007. **PMID: 18174272**.
3. Moore, M. P., Cunningham, R. P., Kelty, T. J., Boccardi, L. R., Nguyen, N. Y., Booth, F. W., & Rector, R. S. (2020). Ketogenic diet in combination with voluntary exercise impacts markers of hepatic metabolism and oxidative stress in male and female Wistar rats. *Appl Physiol Nutr Metab*, 45(1), 35-44. doi:10.1139/apnm-2019-0042. **PMID: 31116955**.
4. Sheldon, R. D., Meers, G. M., Morris, E. M., Linden, M. A., Cunningham, R. P., Ibdah, J. A., Thyfault, J. P., Laughlin, M. H., & Rector, R. S. (2019). eNOS deletion impairs mitochondrial quality control and exacerbates Western diet-induced NASH. *Am J Physiol Endocrinol Metab*, 317(4), E605-E616. doi:10.1152/ajpendo.00096.2019. **PMC6842915**.
5. Puri, P., Baillie, R. A., Wiest, M. M., Mirshahi, F., Choudhury, J., Cheung, O., Sargeant, C., Contos, M. J., & Sanyal, A. J. (2007). A lipidomic analysis of nonalcoholic fatty liver disease. *Hepatology*, 46(4), 1081-1090. doi:10.1002/hep.21763. **PMID: 17654743**.
6. Folch, J., Lees, M., & Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem*, 226(1), 497-509. **PMID: 13428781**.
7. Barrows, B. R., & Parks, E. J. (2006). Contributions of different fatty acid sources to very low-density lipoprotein-triacylglycerol in the fasted and fed states. *J Clin Endocrinol Metab*, 91(4), 1446-1452. doi:10.1210/jc.2005-1709. **PMID: 16449340**.
8. Hellerstein, M. K., & Neese, R. A. (1992). Mass isotopomer distribution analysis: a technique for measuring biosynthesis and turnover of polymers. *Am J Physiol*, 263(5 Pt 1), E988-1001. doi:10.1152/ajpendo.1992.263.5.E988. **PMID: 1443132**.
9. Parks, E. J., Skokan, L. E., Timlin, M. T., & Dingfelder, C. S. (2008). Dietary sugars stimulate fatty acid synthesis in adults. *J Nutr*, 138(6), 1039-1046. doi:10.1093/jn/138.6.1039. **PMC2546703**.
10. Hellerstein, M. K., Neese, R. A., & Schwarz, J. M. (1993). Model for measuring absolute rates of hepatic de novo lipogenesis and reesterification of free fatty acids. *Am J Physiol*, 265(5 Pt 1), E814-820. doi:10.1152/ajpendo.1993.265.5.E814. **PMID: 8238508**.

11. Bederman, I. R., Foy, S., Chandramouli, V., Alexander, J. C., & Previs, S. F. (2009). Triglyceride synthesis in epididymal adipose tissue: contribution of glucose and non-glucose carbon sources. *J Biol Chem*, 284(10), 6101-6108. doi:10.1074/jbc.M808668200. **PMC2649080**.
12. Patton, G. M., & Lowenstein, J. M. (1979). Measurements of fatty acid synthesis by incorporation of deuterium from deuterated water. *Biochemistry*, 18(14), 3186-3188. doi:10.1021/bi00581a042. **PMID: 465462**.
13. Chan, S. C., Liu, C. L., Lo, C. M., Lam, B. K., Lee, E. W., Wong, Y., & Fan, S. T. (2006). Estimating liver weight of adults by body weight and gender. *World J Gastroenterol*, 12(14), 2217-2222. doi:10.3748/wjg.v12.i4.2217. **PMC4087649**.
14. Lambert, J. E., Ramos-Roman, M. A., Browning, J. D., & Parks, E. J. (2014). Increased de novo lipogenesis is a distinct characteristic of individuals with nonalcoholic fatty liver disease. *Gastroenterology*, 146(3), 726-735. doi:10.1053/j.gastro.2013.11.049. **PMC6276362**.
15. Donnelly, K. L., Smith, C. I., Schwarzenberg, S. J., Jessurun, J., Boldt, M. D., & Parks, E. J. (2005). Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J Clin Invest*, 115(5), 1343-1351. doi:10.1172/JCI23621. **PMC1087172**.
16. McDevitt, R. M., Bott, S. J., Harding, M., Coward, W. A., Bluck, L. J., & Prentice, A. M. (2001). De novo lipogenesis during controlled overfeeding with sucrose or glucose in lean and obese women. *Am J Clin Nutr*, 74(6), 737-746. doi:10.1093/ajcn/74.6.737. **PMID: 11722954**.
17. Diraison, F., Moulin, P., & Beylot, M. (2003). Contribution of hepatic de novo lipogenesis and reesterification of plasma non esterified fatty acids to plasma triglyceride synthesis during non-alcoholic fatty liver disease. *Diabetes Metab*, 29(5), 478-485. doi:10.1016/s1262-3636(07)70061-7. **PMID: 14631324**.
18. 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. doi:10.1093/ajcn/81.1.35. **PMID: 15640457**.
19. Matthews, D. R., Hosker, J. P., Rudenski, A. S., Naylor, B. A., Treacher, D. F., & Turner, R. C. (1985). Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia*, 28(7), 412-419. doi:10.1007/BF00280883. **PMID: 3899825**.
20. Katz, A., Nambi, S. S., Mather, K., Baron, A. D., Follmann, D. A., Sullivan, G., & Quon, M. J. (2000). Quantitative insulin sensitivity check index: a simple, accurate method for assessing insulin sensitivity in humans. *J Clin Endocrinol Metab*, 85(7), 2402-2410. doi:10.1210/jcem.85.7.6661. **PMID: 10902785**.
21. Gastaldelli, A., Cusi, K., Pettiti, M., Hardies, J., Miyazaki, Y., Berria, R., Buzzigoli, E., Sironi, A. M., Cersosimo, E., Ferrannini, E., & Defronzo, R. A. (2007). Relationship between hepatic/visceral fat and hepatic insulin

- resistance in nondiabetic and type 2 diabetic subjects. *Gastroenterology*, 133(2), 496-506. doi:10.1053/j.gastro.2007.04.068. **PMID: 17681171.**
22. Angulo, P., Hui, J. M., Marchesini, G., Bugianesi, E., George, J., Farrell, G. C., Enders, F., Saksena, S., Burt, A. D., Bida, J. P., Lindor, K., Sanderson, S. O., Lenzi, M., Adams, L. A., Kench, J., Therneau, T. M., & Day, C. P. (2007). The NAFLD fibrosis score: a noninvasive system that identifies liver fibrosis in patients with NAFLD. *Hepatology*, 45(4), 846-854. doi:10.1002/hep.21496. **PMID: 17393509.**
 23. Vallet-Pichard, A., Mallet, V., Nalpas, B., Verkarre, V., Nalpas, A., Dhalluin-Venier, V., Fontaine, H., & Pol, S. (2007). FIB-4: an inexpensive and accurate marker of fibrosis in HCV infection. comparison with liver biopsy and fibrotest. *Hepatology*, 46(1), 32-36. doi:10.1002/hep.21669. **PMID: 17567829.**
 24. Lee, J. H., Kim, D., Kim, H. J., Lee, C. H., Yang, J. I., Kim, W., Kim, Y. J., Yoon, J. H., Cho, S. H., Sung, M. W., & Lee, H. S. (2010). Hepatic steatosis index: a simple screening tool reflecting nonalcoholic fatty liver disease. *Dig Liver Dis*, 42(7), 503-508. doi:10.1016/j.dld.2009.08.002. **PMID: 19766548.**
 25. Kotronen, A., Peltonen, M., Hakkarainen, A., Sevastianova, K., Bergholm, R., Johansson, L. M., Lundbom, N., Rissanen, A., Ridderstrale, M., Groop, L., Orho-Melander, M., & Yki-Jarvinen, H. (2009). Prediction of non-alcoholic fatty liver disease and liver fat using metabolic and genetic factors. *Gastroenterology*, 137(3), 865-872. doi:10.1053/j.gastro.2009.06.005. **PMID: 19524579.**
 26. McHenry, S., Park, Y., Browning, J. D., Sayuk, G., & Davidson, N. O. (2020). Dallas steatosis index identifies patients with nonalcoholic fatty liver disease. *Clin Gastroenterol Hepatol*, 18(9), 2073-2080 e2077. doi:10.1016/j.cgh.2020.01.020. **PMID: 31982611.**
 27. Long, M. T., Pedley, A., Colantonio, L. D., Massaro, J. M., Hoffmann, U., Muntner, P., & Fox, C. S. (2016). Development and validation of the Framingham steatosis index to identify persons with hepatic steatosis. *Clin Gastroenterol Hepatol*, 14(8), 1172-1180 e1172. doi:10.1016/j.cgh.2016.03.034. **PMC4955680.**
 28. Harrison, S. A., Oliver, D., Arnold, H. L., Gogia, S., & Neuschwander-Tetri, B. A. (2008). Development and validation of a simple NAFLD clinical scoring system for identifying patients without advanced disease. *Gut*, 57(10), 1441-1447. doi:10.1136/gut.2007.146019. **PMID: 18390575.**
 29. Wai, C. T., Greenson, J. K., Fontana, R. J., Kalbfleisch, J. D., Marrero, J. A., Conjeevaram, H. S., & Lok, A. S. (2003). A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. *Hepatology*, 38(2), 518-526. doi:10.1053/jhep.2003.50346. **PMID: 12883497.**
 30. Newsome, P. N., Sasso, M., Deeks, J. J., Paredes, A., Boursier, J., Chan, W. K., Yilmaz, Y., Czernichow, S., Zheng, M. H., Wong, V. W., Allison, M., Tsochatzis, E., Anstee, Q. M., Sheridan, D. A., Eddowes, P. J., Guha, I. N., Cobbold, J. F., Paradis, V., Bedossa, P., Miette, V., Fournier-Poizat,

- C., Sandrin, L., & Harrison, S. A. (2020). FibroScan-AST (FAST) score for the non-invasive identification of patients with non-alcoholic steatohepatitis with significant activity and fibrosis: a prospective derivation and global validation study. *Lancet Gastroenterol Hepatol*, 5(4), 362-373. doi:10.1016/S2468-1253(19)30383-8. **PMC7066580**.
31. Hudgins, L. C., Hellerstein, M., Seidman, C., Neese, R., Diakun, J., & Hirsch, J. (1996). Human fatty acid synthesis is stimulated by a eucaloric low fat, high carbohydrate diet. *J Clin Invest*, 97(9), 2081-2091. doi:10.1172/JCI118645. **PMC507283**.
32. Chong, M. F., Hodson, L., Bickerton, A. S., Roberts, R., Neville, M., Karpe, F., Frayn, K. N., & Fielding, B. A. (2008). Parallel activation of de novo lipogenesis and stearoyl-CoA desaturase activity after 3 d of high-carbohydrate feeding. *Am J Clin Nutr*, 87(4), 817-823. doi:10.1093/ajcn/87.4.817. **PMID: 18400702**.
33. Warensjo, E., Ohrvall, M., & Vessby, B. (2006). Fatty acid composition and estimated desaturase activities are associated with obesity and lifestyle variables in men and women. *Nutr Metab Cardiovasc Dis*, 16(2), 128-136. doi:10.1016/j.numecd.2005.06.001. **PMID: 16487913**.
34. Sjogren, P., Sierra-Johnson, J., Gertow, K., Rosell, M., Vessby, B., de Faire, U., Hamsten, A., Hellenius, M. L., & Fisher, R. M. (2008). Fatty acid desaturases in human adipose tissue: relationships between gene expression, desaturation indexes and insulin resistance. *Diabetologia*, 51(2), 328-335. doi:10.1007/s00125-007-0876-9. **PMID: 18030445**.
35. Knebel, B., Fahlbusch, P., Dille, M., Wahlers, N., Hartwig, S., Jacob, S., Kettel, U., Schiller, M., Herebian, D., Koellmer, C., Lehr, S., Muller-Wieland, D., & Kotzka, J. (2019). Fatty liver due to increased de novo lipogenesis: Alterations in the hepatic peroxisomal proteome. *Front Cell Dev Biol*, 7, 248. doi:10.3389/fcell.2019.00248. **PMC6823594**.

**Chapter III - First-in-class fatty acid synthase inhibitor TVB-2640
reduces hepatic de novo lipogenesis in males with metabolic
abnormalities**

ABSTRACT

Elevated hepatic de novo lipogenesis (DNL) is a key distinguishing characteristic of nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH). In rodent models of NAFLD, treatment with a surrogate of TVB-2640, a pharmacological inhibitor of FAS (FASi), has been shown to reduce hepatic fat and other biomarkers of DNL. The purpose of this Phase I clinical study was to test the effect of the TVB-2640 in obese men with certain metabolic abnormalities that put them at risk for NAFLD. Twelve subjects (mean \pm SE, 42 \pm 2y, BMI 37.4 \pm 1.2 kg/m², glucose 103 \pm 2 mg/dL, TAG 196 \pm 27 mg/dL, and elevated liver enzymes) underwent 10 days of treatment with TVB-2640 at doses ranging from 50-150 mg/d. Food intake was controlled throughout the study. Hepatic DNL was measured before and after an oral fructose/glucose (F/G) bolus using isotopic labeling with 1-¹³C₁-acetate IV infusion, followed by measurement of labeled VLDL-palmitate via GC/MS. Substrate oxidation was measured by indirect calorimetry. Across the range of doses, fasting DNL was reduced by up to 90% ($P=0.003$). Increasing plasma concentrations of TVB-2640 were associated with progressive reductions in the percent of fructose-stimulated peak fractional DNL ($r= - 0.865$, $P=0.0003$) and absolute DNL AUC 6h post F/G bolus ($r= - 0.639$, $P=0.025$). For all subjects combined, ALT was reduced by 15.8 \pm 8.4% ($P=0.05$). Respiratory quotient data was unchanged and safety monitoring revealed that the drug was well tolerated, without an increase in plasma triglycerides. Alopecia occurred in two subjects (reversed after stopping the drug), but otherwise, no changes were observed in fasting glucose, insulin,

ketones, or renal function. These data support the therapeutic potential of FASi, TVB-2640 in particular, in patients with NAFLD and NASH.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) includes a spectrum of symptoms ranging from accumulation of lipids into the liver (steatosis) to inflammation (steatohepatitis, NASH), fibrosis, and cirrhosis (1). The epidemic of NASH and NAFLD continues to grow worldwide. As NASH is being recognized as a major cause of cirrhosis in the U.S. (2), NAFLD is predicted to become a leading cause (3) and replace viral hepatitis as the primary cause of the end-stage liver disease (4). Several studies have identified different metabolic abnormalities associated with NAFLD (1, 5), and among those, increased hepatic de novo lipogenesis (DNL) has been recognized as a key characteristic of NAFLD patients (6, 7). Hepatic DNL is the process by which carbohydrates (glucose, fructose) are made into fat in the liver. We have previously shown that in obese subjects with NAFLD, ~26% of triacylglycerols accumulating in the liver are the product of DNL (7). To aid in the development of therapies targeted for the prevention and treatment of NAFLD some investigations have suggested a central role of transcription factors like the sterol regulatory element-binding (SREBP1c), the liver X receptor, and the carbohydrate receptor element-binding proteins (ChREBP) in the pathogenesis of NAFLD (8-11), while others have pointed toward alterations in lipogenic enzymes as precipitating events (8, 11, 12).

In this regard, studies utilizing rodent models with deletions of lipogenic enzymes such as acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), elongases, stearoyl-CoA desaturase-1, glycerol-3-phosphate acyltransferase (GPAT), and

diacylglycerol O-acyltransferase (DGAT) have demonstrated successful reversal of metabolic abnormalities associated with NAFLD (13-18). Based on these preclinical findings, a number of these key enzymes have been targeted as promising targets of drug development. Early-stage clinical trials using ACC inhibitors have already been completed (19-21), while clinical trials involving DGAT inhibitors are currently ongoing (PF-06865571; NCT03513588). Stiede et al conducted a randomized, controlled trial showing that increasing doses of ACC inhibitor (NDI-010976, GS-0976) progressively reduced fractional DNL (21). Further, in two different studies, Lawitz et al (20) and Loomba et al (19) tested the same ACC inhibitor (GS-0976, 5mg and 20mg respectively) for 12 weeks in patients with NASH. Lawitz reported significant reductions in fasting DNL and Loomba observed reductions in liver fat. However, one of the major concerns with ACC inhibitor treatment was the finding of increased concentrations of plasma triacylglycerols (TAG) (19). FAS is another key enzyme in the DNL pathway and even though pre-clinical studies in knockout mouse models of FAS demonstrated reduced DNL and increased malonyl-CoA (15, 22), no previous clinical trials have been completed in humans examining the impact of FAS inhibition on DNL.

In the present investigation, TVB-2640, a highly-potent (IC_{50} -0.05 μ M), selective and reversible FAS inhibitor (FASi) was administered orally (23, 24). In a past study using a high-fat, high-fructose fed murine model, treatment with the drug's analog prevented hepatic steatosis, inflammation, and fibrosis (25, 26). Further,

in a cohort of cancer patients, the FASi reduced a systemic marker of DNL, serum tripalmitin (27), and also reduced skin DNL, as assessed by sapienic acid (16:1n10), a major component of human sebum (28). However, this drug had not been tested for its direct effect on the pathway of DNL. Therefore, the purpose of this present investigation was to identify a safe dose of TVB-2640 that reduced hepatic DNL in obese men with metabolic abnormalities.

METHODS

The study drug, TVB-2640, was approved for use as an investigational drug by the Food and Drug Administration (IND 132646). All methods and procedures were approved by the University of Missouri Institutional Review Board (MU-IRB# 2006432), and the study registered at ClinicalTrials.gov (NCT02948569). As shown in **figure 3.1**, 95 subjects who responded to an advertisement were contacted by phone to determine preliminary eligibility and seventeen subjects participated in the informed consent process and were screened in person. For screening purposes, fasted (10h) blood draws and anthropometrics were obtained. In addition, questionnaires on alcohol intake and physical activity were administered, as was urine drug screen (Alere™ i Cup® Dx 14, Catalog # I-DX-1147-022, Alere Technology Services, Portsmouth, VA). Two subjects were determined to be ineligible after the screening visit, two subjects did not respond further following the screening visit, and one subject dropped out immediately after the baseline (BL) visit before starting the drug treatment due to unwillingness to perform the magnetic resonance imaging (MRI). As planned, twelve subjects began and completed the study.

Inclusion and exclusion criteria

The inclusion criteria included male sex, age 35-60y, no use of tobacco products, sedentary to moderately active, BMI between the range of 27.1 to 45.0kg/m², elevated alanine aminotransferase (ALT, 42-126 U/L) but below three-times the upper limit of the normal range, family history of cardiovascular disease or type 2

diabetes, willingness to consume the provided isocaloric diet during the study, and maintenance of physical activity. Because a significant concern exists for the exposure of an embryo/fetus to an investigational drug, women of childbearing potential were excluded from this study. Subjects were recruited to have at least one characteristic of the metabolic syndrome (29) or insulin resistance, as evidenced by fasting insulin ≥ 10 mU/L and/or HbA1c within $\geq 4.0\%$ to $\leq 5.6\%$ (30). Subjects were excluded if they had a history of active psychiatric disease, clinically-significant abnormalities on laboratory results, creatinine clearance ≤ 80 mL/min, possessed contraindications to MRI, use of medications for chronic diseases, consumed alcohol > 56 g/week, had major surgery within the past year, or donated 500 ml of blood 8-wks prior to starting the study.

Study design

Subjects were asked to complete three-day food records to characterize their diet which was analyzed via NDSRTM software (University of Minnesota, Minneapolis, Minnesota). These data were used to assess food preferences and prepare a 13-day diet in order to maintain the subject's body weight during the study (**supplementary table S1**). As shown in **figure 3.2**, the diet was provided 3 days prior to a baseline (BL) inpatient test and was continued through the 10-days of drug-treatment. Dietary intake (mean \pm SE) was 3213 ± 168 kcal/d, $36 \pm 1\%$ fat, $17 \pm 0\%$ protein, $48 \pm 1\%$ CHO ($20 \pm 1\%$ of total energy from sugars). Decisions regarding the dose escalation scheme were made by the research safety monitoring team. Subjects received either 50mg, 100mg, or 150mg of TVB-2640

to be taken orally once per day at bedtime. The first six subjects received a 50mg/d dose and as planned a priori, the determinations of the next dosages were made based on the results of the first six subjects. Due to no observed adverse effects in the first six subjects and the effect on DNL, the next two subjects received 150mg/d of the TVB-2640. Subjects who received 150mg exhibited mild side effects, so the last four subjects received 100mg/d of the TVB-2640.

Study visits

As shown in **figure 3.2a**, at BL and after 10d of treatment (post-treatment), subjects participated in a 24h inpatient study. As shown in **figure 3.2b**, subjects were admitted to the University of Missouri Clinical Research Center at 4:00 PM. Vital signs were recorded and a FibroScan® 530 Compact (Echosens™ North America, Waltham, MA) was performed to measure liver steatosis assessed using Controlled Attenuation Parameter (CAP™). Liver stiffness was assessed by FibroScan Vibration-Controlled Transient Elastography (VCTE) at baseline. Values after treatment were considered inaccurate due to the short 10-day duration of the study. Specifically, a reduction in liver fat can lead to a measured reduction in liver stiffness (independent of a change in fibrosis) due to shrinkage of the liver. MRI scan was performed by a radiologist, as previously published (31), to measure intrahepatic triacylglycerols (IHTG) using proton density fat fraction on a Siemens 3T Skyra (series #45839) analyzed by LiverLab software (Siemens Healthcare GmbH, Erlangen, Germany). Intravenous lines were

placed in each antecubital vein – one for infusion of 1-¹³C₁-sodium acetate and the other for blood drawing. Changes in skin surface lipid production (sebum) were assessed using the Sebutape patch (S100, S121, CuDerm Corporation, Dallas, TX). A standardized dinner (pasta with grilled chicken, tomato sauce, and parmesan cheese) was provided at 7:00 PM on day 1 which contained 39% of the subject's total daily energy needs, 25% as fat, 25% protein, and 50% CHO (sugars represented 12% of total daily energy needs). A low-fat, high-carbohydrate dinner was provided the night before to reduce intestinal lipid storage overnight, ensuring that TAG -rich lipoproteins secreted the next morning would be predominantly from the liver (VLDL) and not from the intestine (chylomicron) (32). Subjects slept/rested through the night and the next day at 8:00 AM, an oral fructose/glucose tolerance test (F/GTT) was administered. This consisted of a mixture of 0.9g/kg body weight (BW) of fructose and 0.3g/kg BW dextrose (75:25 ratio wt:wt) dissolved in 180 ml water. Hawkins et al describe how catalytic amounts of fructose may activate glucose phosphorylation (33) and we have shown previously the addition of fructose to glucose increases DNL significantly above glucose alone (34). One gram of Kool-Aid powder (non-caloric lemonade flavor©, 2018 Kraft Foods, Inc., Chicago, IL) was added to the solution to increase the palatability. The prepared drink was served with 100g of ice and subjects asked to consume it within 5min. Blood draws were taken at 10 min prior to the drink and afterward at 15min, 30min, 45min, 1h 15 min, 2h 45 min, 3h 20 min, 3h 40 min, 4h 10 min, and 5h. Measurements of energy expenditure and respiratory quotient were performed at 6:00 AM (fasted) and

10:00 AM (post-sugars) using a Parvo Medics metabolic cart (MMS-2400, Parvo Medics, Murray, Utah, USA). At 2:00 PM body composition was measured using dual x-ray emission absorptiometry (Hologic A version 13.5.2, Marlborough, MA). A standardized lunch was then provided and the subject was discharged from the unit.

After the BL visit, the subject was given 9 doses of the drug to be taken at 10:00 PM each night. For the post-treatment visit after 10 days of drug administration, the subject took their last dose of the drug at 10:00 PM on day 10 of the follow-up inpatient study. Subsequently, blood draws were taken overnight until the next day to measure the drug's pharmacokinetics. TVB-2640 was precipitated from human plasma with acetonitrile and measured using high-pressure liquid chromatography and mass spectrometry (HPLC/MS/MS). An aliquot of the extract was injected onto an HPLC/MS/MS triple quadrupole mass spectrometer. The peak area of the product ion of the TVB-2640 was measured against the peak area of the product ion of the internal standard. A calibration curve spanning the curve range and containing at least six concentrations in duplicate were used to quantify the analyte concentration. The retention times for the TVB-2640 and the internal standard were approximately 1.6 minutes. This quantitative method has a lower limit of quantitation of 2.0ng/mL. Plasma samples were collected over 16h and a 24h AUC concentration/time was extrapolated. In addition to the inpatient studies, four safety visits were performed at 2 days, 4 days, and 7 days during treatment (**figure 3.2b**). During

these visits, subjects were queried for potential adverse effects of the drug using a questionnaire. Blood was drawn for the measurement of liver enzymes (aspartate aminotransferase, AST, and ALT) and glucose concentrations. During treatment at day 7, the skin sebum collection was also performed. Within one week after completion of drug treatment, the final safety visit (post 6d) was conducted. Two subjects who reported transient alopecia during treatment were also seen for monitoring at 2, 4, and 6-weeks after the end of treatment.

Primary and secondary outcomes

The putative effects of TVB-2640 are presented in **figure 3.2c**. The primary outcomes of this study were the assessment of drug safety and changes in DNL (hypothesized to be significantly reduced). A secondary outcome was a change in liver fat mediated by the metabolic pathways shown in **figure 3.2c**. Other secondary outcomes were sebum production and changes in blood pressure (BP). Peripheral BP was measured using a Philips, SureSigns S53 Sphygmomanometer (Andover, MA) and data represent the average of three measurements taken one minute apart. Other measurements included concentrations of HbA1c, plasma lipids, glucose, insulin, ketones, lactate, apolipoprotein B100 (apoB100) in very low-density lipoproteins (VLDL), and nonesterified fatty acids (NEFA).

Analytical methods

$1\text{-}^{13}\text{C}_1$ -Sodium acetate was purchased from Cambridge Isotope Laboratory, Inc. (99%, microbiological/pyrogen tested, Tewksbury, MA). The isotope infusion was prepared at the University of Missouri Hospital's Investigational Pharmacy using Good Clinical Practice requirements under a laminar flow bench and was made up of 10g of $1\text{-}^{13}\text{C}_1$ -sodium acetate was dissolved in 1L of 0.5% saline as described previously (34, 35). During the inpatient visit, the $1\text{-}^{13}\text{C}_1$ -sodium acetate infusion was started at 6:00 PM at a continuous rate of 0.8mL/min (48mL/hr) for 14h (**figure 3.2b**). From plasma, VLDL-TAG particles were immediately isolated by ultracentrifugation at 40,000 rpm in a 50.3Ti rotor (Beckman Instruments, Palo Alto, CA) for 20h at 15°C (36), TAG isolated and fatty acid methyl esters prepared as described previously (36). Labeled palmitate was measured using a 6890N gas chromatography coupled to a 5975 mass spectrophotometry detector (Agilent Technologies, Palo Alto, CA) and DNL calculated by mass isotopomer distribution analysis (37). The fatty acid composition was measured by a 6890N gas chromatography system (Agilent Technologies, Palo Alto, CA). Fractional DNL was calculated using the MIDA method (6). Absolute VLDL DNL-palmitate (16:0) was calculated by multiplying the fractional DNL by the quantity of VLDL-TAG that was palmitate, as measured via GCMS. Here, palmitate is used as a surrogate for new fatty acids in TAG (6, 7, 34, 35, 38), as a significant proportion of saturated (39) and monounsaturated fatty acids are produced through this pathway (40), the percentage of palmitate increases 2-3 fold with sugar consumption, and the percentage of palmitate is

directly correlated with DNL (41). Sebum production was assessed via Sebutape analysis (S100, S121, CuDerm Corporation, Dallas, TX) according to the manufacturer's directions. Accordingly, skin oils were collected by placing the Sebutape on the forehead for 30 min, lipids were extracted and analyzed by Metabolon, Inc. (Morrisville, NC).

Plasma concentrations of total cholesterol, TAG, low-density lipoprotein cholesterol (LDLc), HDLc, AST, and ALT were measured by a CLIA-standardized laboratory (Boyce and Bynum Pathology Laboratory, Columbia, MO, 26D0652373). The measurements of lipids were performed via auto-analyzer (Roche Cobas 8000 System, CV 0.6-0.9%, Indianapolis, IN) using electrochemiluminescent immunoassay. Liver enzymes were measured using UV Absorbance (Roche Cobas 8000 System, CV 0.5-3.2% for AST and 0.5-3.1% for ALT, Indianapolis, IN). Assay kits were used to measure the concentrations of plasma glucose (Wako #439-90901, CV 6.6%, Mountain View, CA) and NEFA (Wako #991-34891, CV 6.9%, Mountain View, CA). Plasma insulin was measured using an enzyme-linked immunosorbent assay (Human Insulin, EMD Millipore #EZHI-14K, CV 7.2%, Billerica, MA) and plasma ketones, using a cyclic enzymatic assay (Wako #415-73301 R1, 411-73401 R2, CV 1.34-1.92%, Mountain View, CA). Plasma lactate was measured using YSI 2300 Stat Plus Glucose & Lactate Analyzer (Yellow Springs, Ohio). VLDL-apo B100 was measured by ELISAPRO kit (Human apoB, Mabtech, Inc # 3715-1HP-2, CV 2%,

Cincinnati, OH). The amount of VLDL-TAG per particle was calculated as the molar ratio of VLDL-TAG per VLDL-apoB100 (mol/mol).

Statistical analysis and calculations

StatView®, 5.0.1 software (v2008) was used when a paired sample t-test was performed to test the relationship between baseline and follow-up data for all subjects combined. Using the statistical package for the social sciences (SPSS®, v24, 2016), regression analysis was performed to test the relationship between the dosage of the drug and DNL and drug dose and IHTG, and DNL and IHTG. One-factor analysis of variance (ANOVA) was performed to test the difference between pharmacokinetic response of each dose. Two-factor ANOVA, and Holm-Sidak post hoc analyses were performed using dose as between-subject variable and time (post-administration in case of fructose/glucose bolus and 10-days treatment) as within-subject variable to test the effect of drug on variables measured. Pearson correlation analysis was performed using SPSS® (v24, 2016). HOMA-IR was calculated as [(glucose in mg/dL*insulin in μ U/mL)/405].

RESULTS

Subject anthropometrics and the pharmacokinetics of TVB-2640

No differences were found in the subject characteristics among the dosing groups (**table 1**). The age (mean±SE) of all subjects combined was 42±2y. The subjects were overweight (121±5 kg and BMI 37.4±1.2 kg/m²) and had elevated plasma glucose (103±2mg/dL) and (HbA1c 5.7±0.1%). Liver enzymes were elevated and within three times the upper limit of the normal range. Baseline liver fat was 10.0±2.4% by MRI, 317±20 dB/m by FibroScan™, and liver stiffness (E) was 11.6±3.1 kPa. The subjects' body weights were maintained throughout treatment, as expected (**supplementary figures S1a-c**). With regard to the steady-state (10d) pharmacokinetics of TVB-2640, **figure 3.3a** presents the plasma concentrations of the drug for each dose, and **figure 3.3b** represents the calculated 24-h area-under-the-curve (AUC₀₋₂₄) for each dose. The AUC₀₋₂₄ was dose-dependent and was different for 100mg (P=0.071) and 150mg (P=0.014) compare to AUC₀₋₂₄ for 50mg dose. The half-life (t_{1/2}) of the drug, determined from a larger dataset of results from other studies of TVB-2640 in humans, has been found to be between 10-14 h which is in line with other studies of this drug (42).

De novo lipogenesis, plasma lipids, and liver fat

As shown in **figure 3.4a**, the fractional level of DNL in the fasting state, measured before the F/G bolus was given, was significantly inhibited with the TVB-2640 100 mg (P≤0.001) and 150 mg dose (P≤0.001) but not with 50mg

dose ($P=0.220$). For absolute DNL (**figure 3.4b**), TVB-2640 significantly inhibited fasting DNL with the 150mg dose ($P<0.001$) and 100mg ($P=0.036$), and no change was found with the 50mg dose ($P=0.544$). Interestingly, inhibition of DNL-16:0 occurred without changes in VLDL-16:0 (**figure 3.4c**) or plasma TAG (**figure 3.4d**) for all three groups. These data, along with the number of TAG molecules per particle and the lack of change in VLDL-apoB100 (either fasting or the peak after the sugars bolus, **table 3.2**) suggest that another source of fatty acids may have been used to support lipoprotein assembly. With regard to the acute effect of sugars, BL fractional DNL, and absolute DNL-16:0 were significantly elevated after the F/G bolus (**figure 3.4a & b**). Following 10d of treatment, the F/G bolus-induction was inhibited; DNL stimulation was 23% lower after 50mg/d (**figure 3.5a**, $P=0.033$), 65% lower after 100mg/d ($P=0.008$), and 77% lower after 150mg/d ($P<0.001$). The IHTG analyzed by MRI using proton density fat fraction was significantly reduced on average from 10.0% to 8.4% (**figure 3.5b**, t-test, $P=0.016$, ANOVA=0.048) and this reduction in IHTG primarily appeared to be driven by the subjects who were treated with 100mg/d ($P=0.06$). Liver fat, as assessed via FibroScan CAP score, was significantly reduced in the 100mg/d ($P=0.025$) and 150mg/d dose groups ($P=0.004$, **figure 3.5c**). As shown in **figures 3.5d-f**, reductions in peak absolute stimulation of DNL (**figure 3.5d**) and IHTG (**figure 3.5e**) were inversely associated with the drug's AUC_{0-24h} ($P=0.0003$ and $P=0.029$, respectively). Further, the reduction in fractional DNL was significantly associated with a reduction in the liver fat percent (**figure 3.5f**).

Blood cholesterol and ketosis

Prior studies of ACC inhibitors have shown increased TAG concentration with a decrease in PUFA composition of TAG . As shown in **figure 3.6a**, neither plasma TAG nor VLDL-TAG changed although concentrations were highly variable after 10-days of TVB-2640. No reductions in PUFA were found in the present study (**supplementary figures S3a-c**). All forms of cholesterol i.e., total cholesterol (P=0.010) and LDLc (P=0.003) were significantly reduced in subjects who took 100mg/d, whereas no significant changes were observed in the other groups. HDLc was significantly reduced in subjects who took 50mg (P=0.001) and 100mg (P=0.036) but not in the two subjects who took 150mg (P=0.139, **figure 3.6a**). We found no differences in ketone concentrations which is in contrast to past observations of ketosis with ACC inhibitor (13). As shown in **table 3.2**, no changes were observed in respiratory quotient (fasting or fed), glucose oxidation, or fatty acid oxidation for all three groups. Further, no changes were observed for plasma lactate and blood CO₂ in all three groups.

Drug safety and adverse drug reactions

With regard to drug safety, during and after completion of the study, ALT concentrations were significantly reduced, whereas no changes were observed in plasma AST levels (**figures 3.6b-c**). As shown in **supplementary table S2**, systolic BP did not change (ANOVA, P=0.413) but diastolic BP reduced significantly (ANOVA, P=0.045). No changes were observed in fasting and fed glucose and insulin concentrations with all three doses (**supplementary figure**

S2a-b). Similarly, fasting NEFA was not different for all three groups but the rebound effect on postprandial NEFA was significantly lower with the 100mg/d dose at 3.5h, 5h, and 6h (**supplementary figure S2c**). When AUC values were calculated for these biochemical measurements in response to F/G bolus, NEFA AUC values were significantly lower after treatment with 150mg/d dose. Few adverse drug reactions were reported with each dose of the drug (**supplementary table S3**). One subject in the 50mg/d group reported dry skin and one subject in the 100mg/d group reported dry mouth; both symptoms disappeared upon completion of drug treatment. Two subjects (one treated with 100mg and one treated with 150mg) reported mild and transient alopecia. Following the completion of treatment, these subjects were seen for 6wks, at 2wk intervals and hair loss was documented to have recovered. **Supplementary figures S4a-b** present the sebum analysis for these two subjects. TAG 48:0 and TAG 48:1 make up approximately 50% of all TAG in sebum. The 16:1 fatty acid in TAG 48:1 is most likely sapienic acid (16:1, n-10) which is unique to sebum (43, 44). Concentrations of TAG species 48:0 and TAG 48:1 fell at 6d and 10d of treatment and began to recover 2 weeks after completion of treatment. Total TAG in sebum and the top 50 individual TAG for these two subjects are presented in the **supplementary figures S4c-e**. Lastly, in addition to the symptoms that are listed in the **supplementary table S3**, subjects were also asked to report any other reactions they may have experienced during the treatment. None of the subjects reported other complications, including ophthalmological complications.

DISCUSSION

The primary finding of the current investigation is that 10d of treatment with TVB-2640, an inhibitor of the β -ketoacyl reductase domain of the fatty acid synthase enzyme (FAS) complex, reduced hepatic de novo lipogenesis (DNL) and decreased intrahepatic lipid content (IHTG) in male subjects with characteristics of metabolic syndrome. Specifically, increasing drug doses suppressed DNL in a stepwise fashion such that at 150mg/d, DNL appeared to be fully suppressed when stimulated by the consumption of a bolus of liquid sugars. The maximum absolute reduction in IHTG at 10 days was 4.2% and this effect was found predominantly in those subjects who received 100mg/d dose. However, it is uncertain whether the 50mg/d dose was less effective because of the amount of drug or because the subjects who received this dose started with lower levels of liver fat at the beginning of the study, compared to 100mg/d group. Further, in the 100mg/d dose group, concentrations of total cholesterol, LDLc, and HDLc were reduced. Among the three sources of fatty acids that can contribute to IHTG (adipose, diet, and DNL), increased DNL has been shown as a key contributor to the pathogenesis of NAFLD in subjects with insulin resistance (6, 7). In this study, the treatment decreased IHTG but VLDL-TAG and apo-B100 did not change. Thus, the primary mechanisms for the decrease in IHTG found here were likely a direct effect of inhibiting the lipogenesis pathway (28). With these changes, ALT and AST were significantly reduced. These data highlight the potential role of the FAS enzyme to contribute to liver lipid accrual. Treatment did not change fasting NEFA or ketone body concentrations nor

whole-body fatty acid oxidation. Thus, the continued flux of NEFA to the liver also may have supported VLDL-TAG production.

The lack of change in both VLDL- and plasma-TAG in the present human study are consistent with a preclinical study in mice in which TAG levels did not change with the complete knockout of the FAS enzyme (45). A recent study in a diet-induced, obese murine model of NASH showed a decrease of plasma-TAG with a drug similar to TVB-2640 (25). By contrast, targeting hepatic lipid synthesis through acetyl-CoA carboxylase inhibition (ACCi) in humans and rodents consistently raises VLDL- and plasma-TAG concentrations (13, 19, 20, 46). For example, in an open-label prospective study, Lawitz et al administered an ACC inhibitor (ACCi GS 0976) to patients with NASH and reported lower DNL and IHTG contents accompanied by non-significant increases in plasma-TAG (20). In a study of healthy subjects with hepatic steatosis, Kim et al found that inhibition of ACC with the compound MK-4074 reduced liver fat but increased fasting VLDL-TAG concentrations (13). Goedeke et al treated Sprague-Dawley rats with an ACCi drug termed 'compound 1' and found significant reductions in liver-TAG content, while plasma-TAG more than doubled (46). These changes were attributed to a 15% increase in VLDL production and a 20% reduction in LPL-mediated TAG clearance. Lastly, Kim et al investigated hepatic lipid synthesis in an ACC knockout mouse and concluded that the observed increase in VLDL-TAG concentration resulted from a reduction in liver PUFA content, a consequence of lower malonyl-CoA levels, and increased expression of

SREBP1c and GPAT leading to increased VLDL production (13). A role for PUFA signaling in these mechanisms is unclear. In the present study, FASi did not elevate plasma-TAG nor reduce PUFA levels in fasting VLDL-TAG .

With regard to safety, as described above, although plasma- and VLDL-TAG responses vary depending on whether ACC or FAS is targeted, inhibition of lipid synthesis causes reductions in liver-TAG content that are consistently associated with reduced plasma ALT concentrations. We were surprised to observe this effect as early as four days and ALT remained lower even after 6 days post-treatment with TVB-2640. Reductions in ALT are consistent with previous studies conducted in both animal models of FASi (15) and in human subjects who were treated with different ACCi (19, 20). The fact that ALT concentrations did not increase above baseline supports the observation that there was no toxic impact on the liver; indeed a significant reduction in ALT suggests liver function improved. Further indications of safety included no change in systolic or diastolic BP, consistent with past literature (47). The transient alopecia observed in two subjects was likely due to a decline in fatty acid production in sebocytes (44). As shown in **supplementary figures S2a & b**, this decline in sebum TAG began to resolve 2wks after cessation of the drug, and evidence of hair regrowth was observed. With regard to LDLc, data from preclinical and primary cell culture demonstrate that FASi decreases proprotein convertase subtilisin/kexin type 9 (PCSK9) and increases expression of the insulin-induced gene 1 (Insig-1), both effects that would lower LDLc (15, 48, 49).

Limitations of this investigation included relatively small size, short duration, and inclusion of only men, common characteristics of Phase 1 studies. Future studies should include subjects with documented NASH treated with TVB-2640 for longer periods of time. The choice of 1-¹³C₁-sodium acetate to measure lipogenesis was made due to the isotope's fast decay rate which accommodates studies of short duration. To be utilized in lipogenesis, acetate requires activation by acetyl coenzyme-A synthase (ACS) and, although highly unlikely, it is possible that the observed reduction in DNL could have been due to an off-target effect of the drug on ACS. To test this possibility, the acetyl-CoA precursor enrichment (p) was used as an indicator of the efficiency of ACS to activate the isotope. Drug treatment did not change the enrichment of the acetyl-CoA precursor pool during the F/GTT (data not shown) and thus, the drug-induced reductions in DNL likely reflect a direct inhibition of FAS. Further support for this was found in the significant correlation between drug concentration and the reduction in liver fat (**figures 3.5e-f**).

In summary, ten days of treatment with TVB-2640 significantly reduced DNL in subjects with characteristics of metabolic syndrome without raising concentrations of blood lipids. These data are consistent with previous studies conducted in animal models with inhibition of FAS (15, 22, 25, 28, 45).

Reductions in DNL acutely decreased liver fat, biochemical markers of liver injury, and cholesterol levels. These effects were observed primarily in subjects who were administered the 100mg/d of dose. Future studies are needed to

determine the appropriate dose and effects of longer-term use.

Table 3.1: Subject characteristics at screening

Subject characteristics	50 mg n=6	100 mg n=4	150 mg n=2	ANOVA*
Age (y)	41 ± 2	44 ± 4	43 ± 5	0.715
Body weight (kg)	124.0 ± 6.0	116.0 ± 10.3	117.3 ± 12.2	0.768
BMI (kg/m ²)	37.4 ± 1.4	37.6 ± 2.9	37.3 ± 3.6	0.994
Waist (cm)	125.0 ± 4.0	122.3 ± 5.6	119.5 ± 9.5	0.808
Plasma glucose (mg/dL)	100 ± 3	109 ± 3	100 ± 2	0.200
HDL (mg/dL)	37 ± 3	44 ± 6	34 ± 0	0.430
Triacylglycerols (mg/dL)	201 ± 32	171 ± 67	230 ± 75	0.799
HbA1c (%)	5.6 ± 0.1	5.8 ± 0.2	5.8 ± 0.2	0.702
Systolic BP (mmHg)	139 ± 6	130 ± 5	123 ± 9	0.268
Diastolic BP (mmHg)	88 ± 5	87 ± 1	81 ± 4	0.614
GGT (U/L)	31 ± 4	55 ± 23	74 ± 43	0.291
eGFR (mL/min/1.7 m ²)	120 ± 9	124 ± 7	116 ± 11	0.872
BUN (mg/dL)	15 ± 1	12 ± 1	12 ± 2	0.160

Legend: Data are mean ± SE. Abbreviations: GGT, gamma-glutamyltranspeptidase; eGFR, estimated glomerular filtration rate; and BUN, blood urea nitrogen. * One-way ANOVA was performed to test for differences between three groups. No differences were found between the groups for any of the variables.

Table 3.2: Biochemical measurements and substrate oxidation before and after 10 days TVB-2640 treatment

Biochemical variables	50 mg (n=6)		100 mg (n=4)		150 mg (n=2)	
	Baseline	Post-treatment	Baseline	Post-treatment	Baseline	Post-treatment
Fasting VLDL-apo B100 (mg/dL)	89 ± 19	101 ± 25	69 ± 36	61 ± 19	108 ± 14	92 ± 9
Peak VLDL-apo B100 (mg/dL)	135 ± 20	126 ± 18	111 ± 44	81 ± 23	127 ± 5	118 ± 13
Fasting VLDL-TG moles per particle	22,216 ± 5,680	14,831 ± 1,457	10,129 ± 3,107	11,361 ± 2,288	13,091 ± 3,436	14,872 ± 834
Peak VLDL-TG moles per particle	31,543 ± 6,975	18,410 ± 1,709	11,263 ± 2,857	12,837 ± 2,954	16,470 ± 165	16,368 ± 228
Plasma ketones (µmol/L)	53 ± 7	52 ± 5	73 ± 16	62 ± 11	56 ± 3	48 ± 1
Plasma lactate (mg/dL)	9.8 ± 1.0	10.0 ± 0.6	9.4 ± 1.5	9.9 ± 1.0	11.0 ± 0.0	11.5 ± 0.4
Blood CO ₂ (mmol/dL)	25.2 ± 0.6	23.7 ± 0.9	23.6 ± 1.1	24.5 ± 0.3	24.5 ± 1.5	23.5 ± 0.5
Substrate oxidation						
Fasting RQ	0.85 ± 0.02	0.85 ± 0.01	0.88 ± 0.02	0.84 ± 0.01	0.89 ± 0.07	0.93 ± 0.04
Fed RQ	0.91 ± 0.01	0.92 ± 0.02	0.90 ± 0.00	0.92 ± 0.01	0.96 ± 0.05	0.90 ± 0.01
Fasting glucose oxidation (mg/kg/min)	1.41 ± 0.20	1.35 ± 0.14	1.72 ± 0.24	1.38 ± 0.13	1.66 ± 0.69	1.96 ± 0.39
Fed glucose oxidation (mg/kg/min)	2.24 ± 0.07	2.23 ± 0.15	2.26 ± 0.11	2.28 ± 0.05	2.57 ± 0.61	1.83 ± 0.15
Fasting fat oxidation (mg/kg/min)	0.58 ± 0.08	0.57 ± 0.04	0.53 ± 0.06	0.62 ± 0.07	0.34 ± 0.27	0.23 ± 0.15
Fed fat oxidation (mg/kg/min)	0.36 ± 0.06	0.31 ± 0.07	0.44 ± 0.03	0.36 ± 0.07	0.11 ± 0.23	0.38 ± 0.05

Legend: Data are mean ± SE. Peak values represent the highest values observed after the oral fructose/glucose tolerance test. VLDL-TG per particle is the calculated molar ratio of VLDL-TG per VLDL-apoB100. A paired-sample t-test was performed to test the effect of treatment in each group. There were no changes detected due to treatment.

Figure 3.1: Consort flow

CONSORT

TRANSPARENT REPORTING of TRIALS

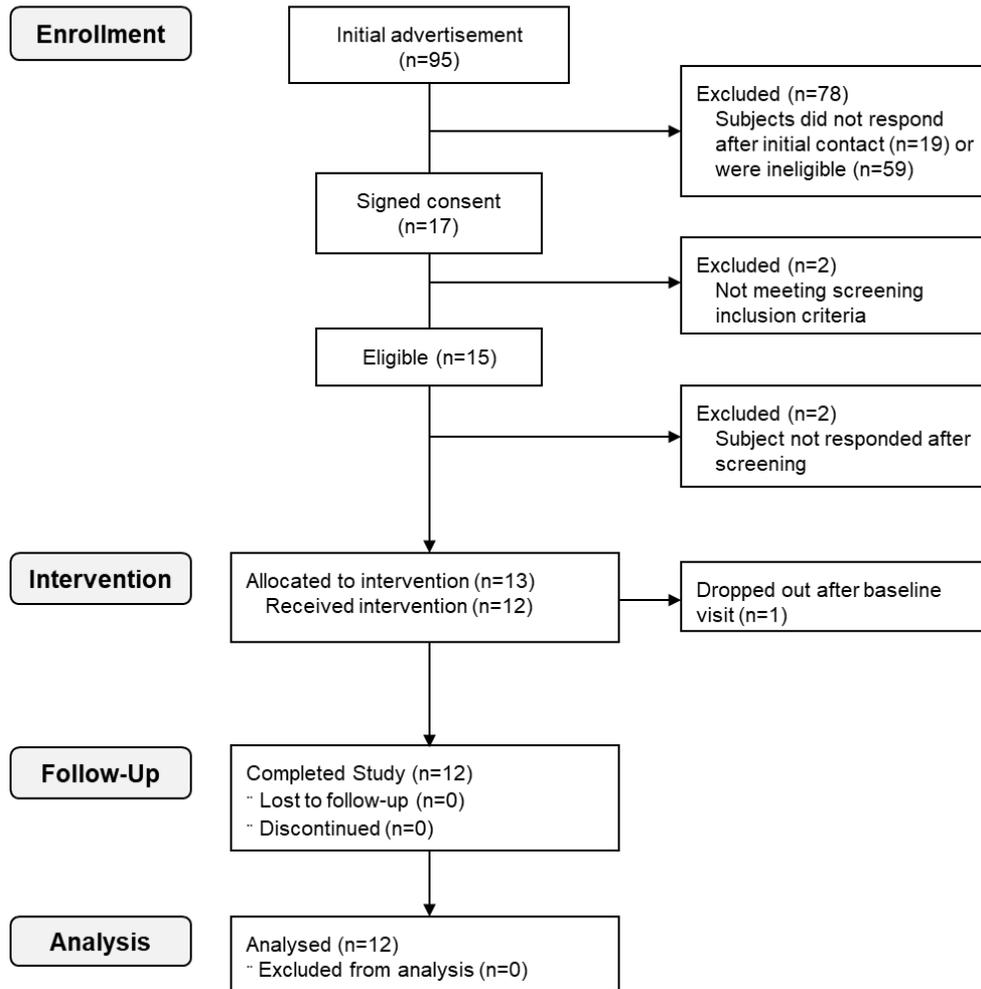
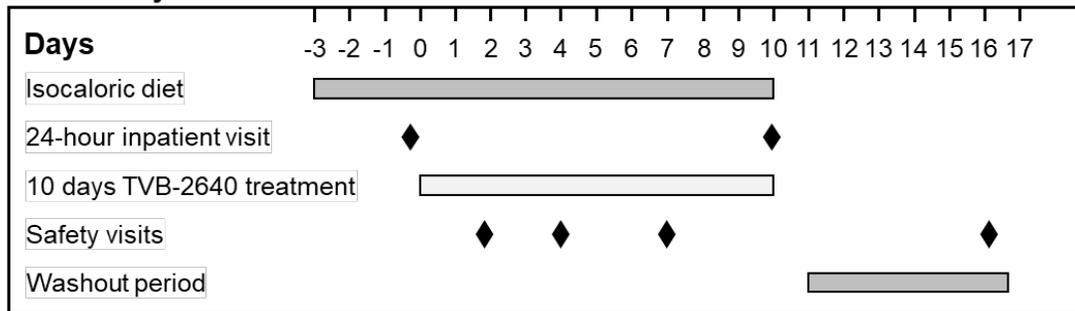
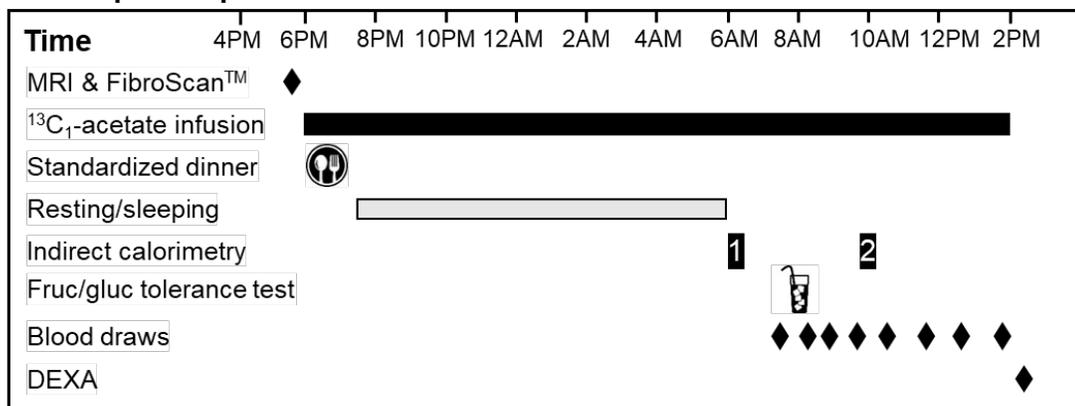


Figure 3.2: Overall study design and in-patient protocol

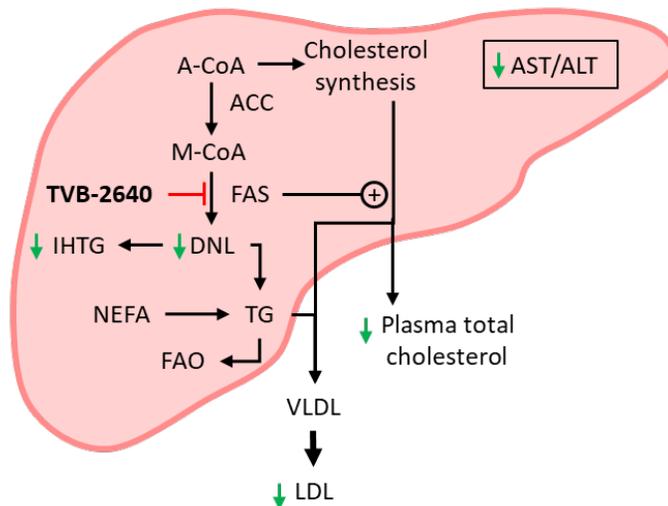
3.2a. Study overview



3.2b. Inpatient protocol



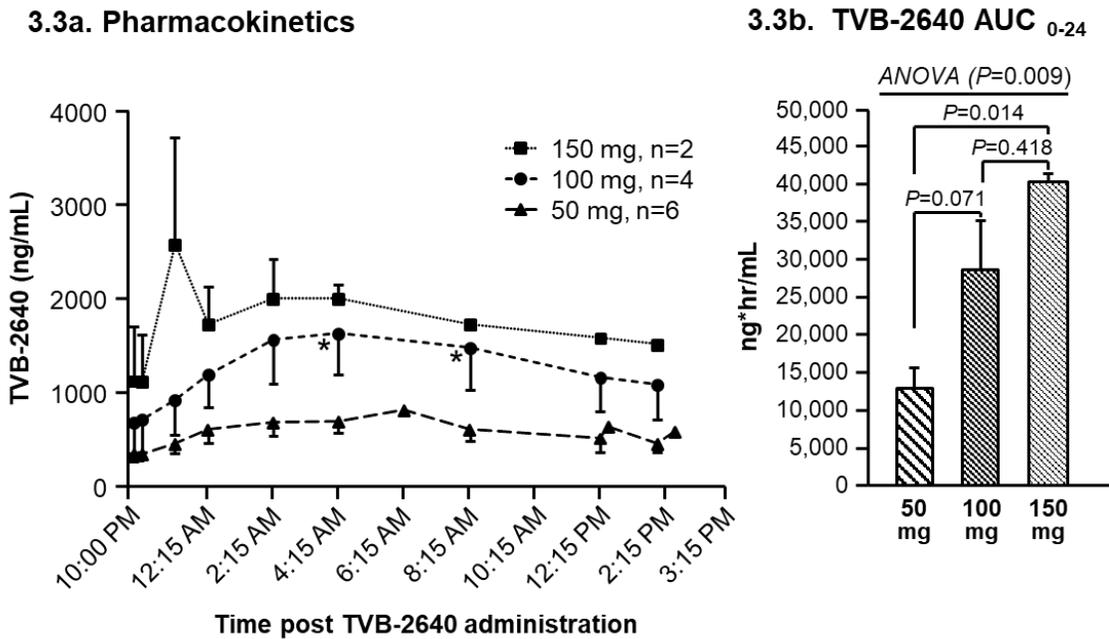
3.2c. Pathways affected by TVB-2640



Legend: **3.2a.** Subjects were treated with TVB-2640 for 10d during which time they consumed a standardized diet and were monitored for side effects (as described in the methods section). **3.2b.** Inpatient metabolic studies were

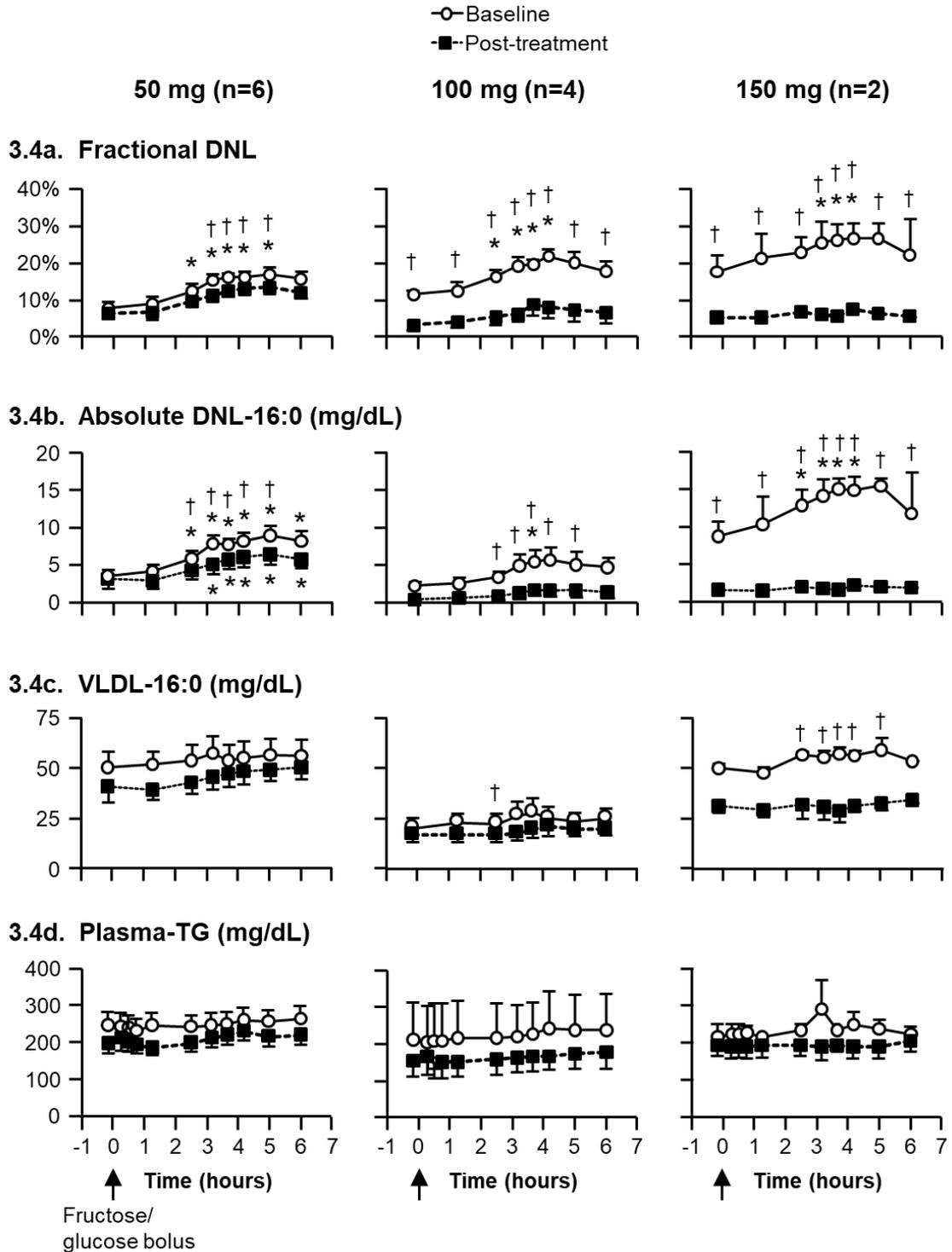
performed before and after 10d of treatment. **3.2c.** Schematic representation of pathways effected by inhibition of FASN enzyme. Abbreviations: MRI, magnetic resonance imaging; DEXA, dual energy x-ray absorptiometry; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DNL, de novo lipogenesis; FAS, fatty acid synthase; IHTG, intrahepatic triacylglycerols; LDL, low-density lipoprotein cholesterol; NEFA, nonesterified fatty acids; TG, triacylglycerol; VLDL, very low-density lipoproteins.

Figure 3.3: Pharmacokinetics and plasma concentration AUC after 10d of dosing



Legend: 3.3a. Dose response curve for three doses of TVB-2640. Repeated measure ANOVA was performed to test the dose response curve for three doses over time. During the analysis, dose was used as a between groups factor and 'time post administration' was used as within groups factor. Concentrations of drug tended to be different between three groups ($P=0.066$). Post hoc test was conducted using Sidak-Holm analysis. Dose response curve for 50 mg group was significantly lower than 150 mg group ($P=0.011$) and tended to be lower than 100 mg group ($P=0.083$). No differences were observed between 100 mg group and 150 mg group ($P=0.328$). * $P<0.05$ compare to baseline value for 100 mg group. **3.3b.** Area-under-the-curve (AUC) for each dose response curve. One way analysis of variance was conducted to test the difference between three doses. A significant difference was found between the three doses ($P=0.009$). Post hoc test was conducted using Sidak-Holm analysis. Significant difference was found between the 50 mg and 150 mg groups ($P=0.014$). Concentrations obtained with 100 mg group tended to be different compared to 50 mg ($P=0.071$) and were not different compared to 150 mg group ($P=0.418$).

Figure 3.4: Changes in liver enzymes, plasma lipids, and liver fat

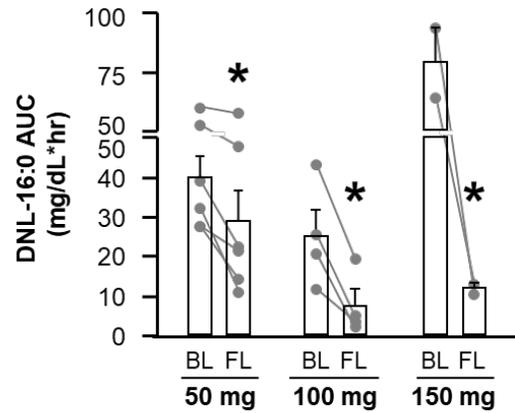


Legend: Data are mean \pm SE. Repeated measure ANOVA was conducted by using two within factors and one between factor. Time post consumption of fructose/glucose bolus was used as a first factor, 10 days treatment was used as a second factor for within factors, and group (dose) was used as a between factor. **3.4a.** Fractional DNL in response to fructose/glucose tolerance test.

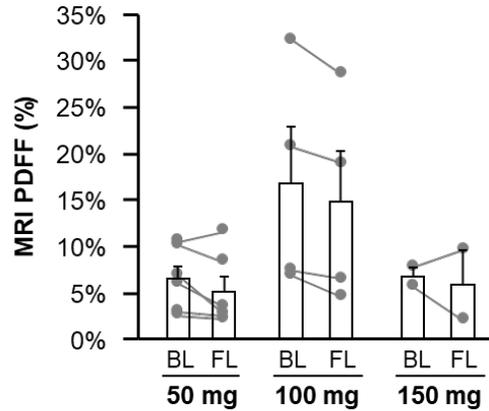
Between factor analysis revealed that fractional DNL was significantly reduced with drug treatment in all subjects combined ($P=0.001$) and the values were lower during follow-up visit compare to baseline values ($P<0.001$). This reduction in fractional DNL was also different between groups ($P=0.007$). Within factor analysis revealed that before the drug treatment, fractional DNL was significantly stimulated with fructose/glucose bolus for all three groups (50 mg, $P=0.005$; 100 mg, $P=0.015$; 150 mg, $P=0.021$). $*P\leq 0.05$ compare to fasting value. After the drug treatment, for all subjects combined, this stimulation of DNL was significantly reduced ($P=0.001$) and therefore, no changes were observed in response to fructose/glucose bolus in all three groups (50 mg, $P=0.256$; 100 mg, $P=0.549$; 150 mg, $P=0.619$). † represents significant difference between baseline and post-treatment at each point. $P\leq 0.05$ for 50 mg group, and $P\leq 0.001$ for 100 mg and 150 mg group. **3.4b.** Absolute DNL-16:0 in response to fructose/glucose tolerance test. Between factor analysis revealed that absolute DNL-16:0 was significantly lower at follow-up compare to baseline values ($P<0.001$). This reduction in absolute DNL-16:0 was tended to be different between groups ($P=0.072$). $*P\leq 0.05$ compare to fasting value. † represents significant difference between baseline and post-treatment at each point. $P\leq 0.05$ for 50 mg and 100 mg group, and $P\leq 0.001$ for 150 mg group. **3.4c.** VLDL-16:0 concentration in response to fructose/glucose tolerance test. For between factor analysis, VLDL-16:0 did not change for all subjects ($P=0.500$) or between groups ($P=0.685$). Within factor analysis revealed that VLDL-16:0 did not change in response to fructose/glucose bolus for all three groups both before (50 mg, $P=0.260$; 100 mg, $P=0.664$; 150 mg, $P=0.630$) and for one group after the drug treatment (50 mg, $P=0.017$; 100 mg $P=0.262$; 150 mg, $P=0.404$). † represents significant difference between baseline and post-treatment at each point ($P\leq 0.05$). **3.4d.** Plasma-TG concentration in response to fructose/glucose tolerance test. For between factor analysis, plasma-TG did not change for all subjects ($P=0.129$) or between groups ($P=0.968$). Within factor analysis revealed that plasma-TG did not change in response to fructose/glucose bolus for all three groups both before (50 mg, $P=0.162$; 100 mg, $P=0.608$; 150 mg, $P=0.131$) and after the drug treatment (50 mg, $P=0.579$; 100 mg $P=0.338$; 150 mg, $P=0.992$). Open circles (○, baseline), closed boxes (■, post-treatment). Abbreviations: TG, triacylglycerols; VLDL, very low density lipoproteins; DNL, de novo lipogenesis.

Figure 3.5: Changes in DNL and correlation analysis between TVB-2640 AUC, DNL, and liver fat

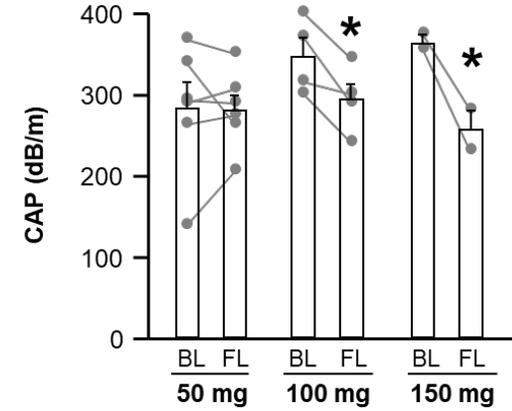
3.5a. De novo lipogenesis



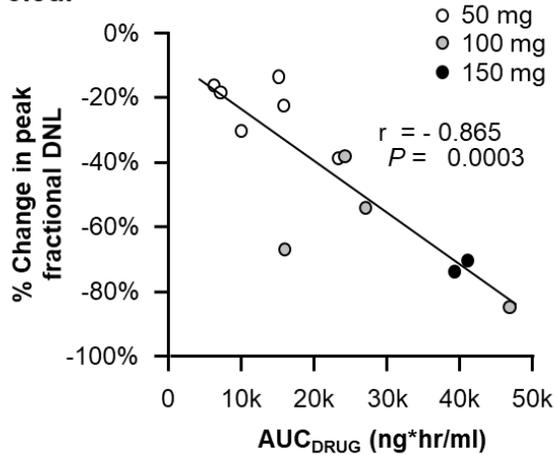
3.5b. Intrahepatic Triglycerides



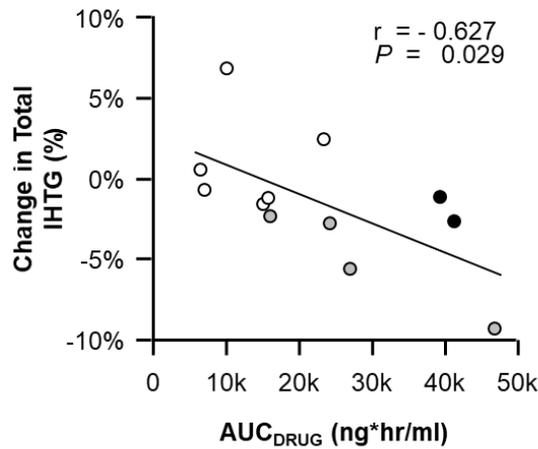
3.5c. FibroScan Liver Fat



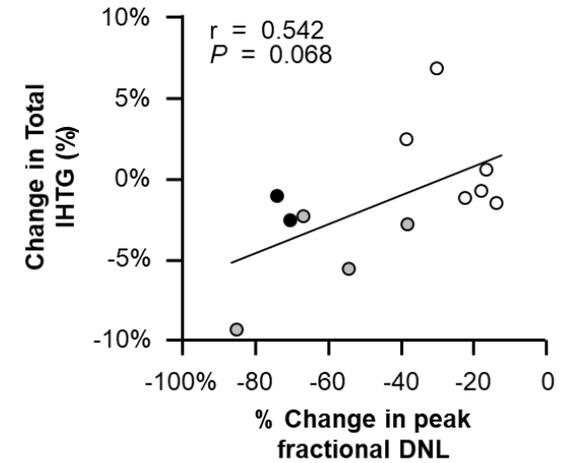
3.5d.



3.5e.



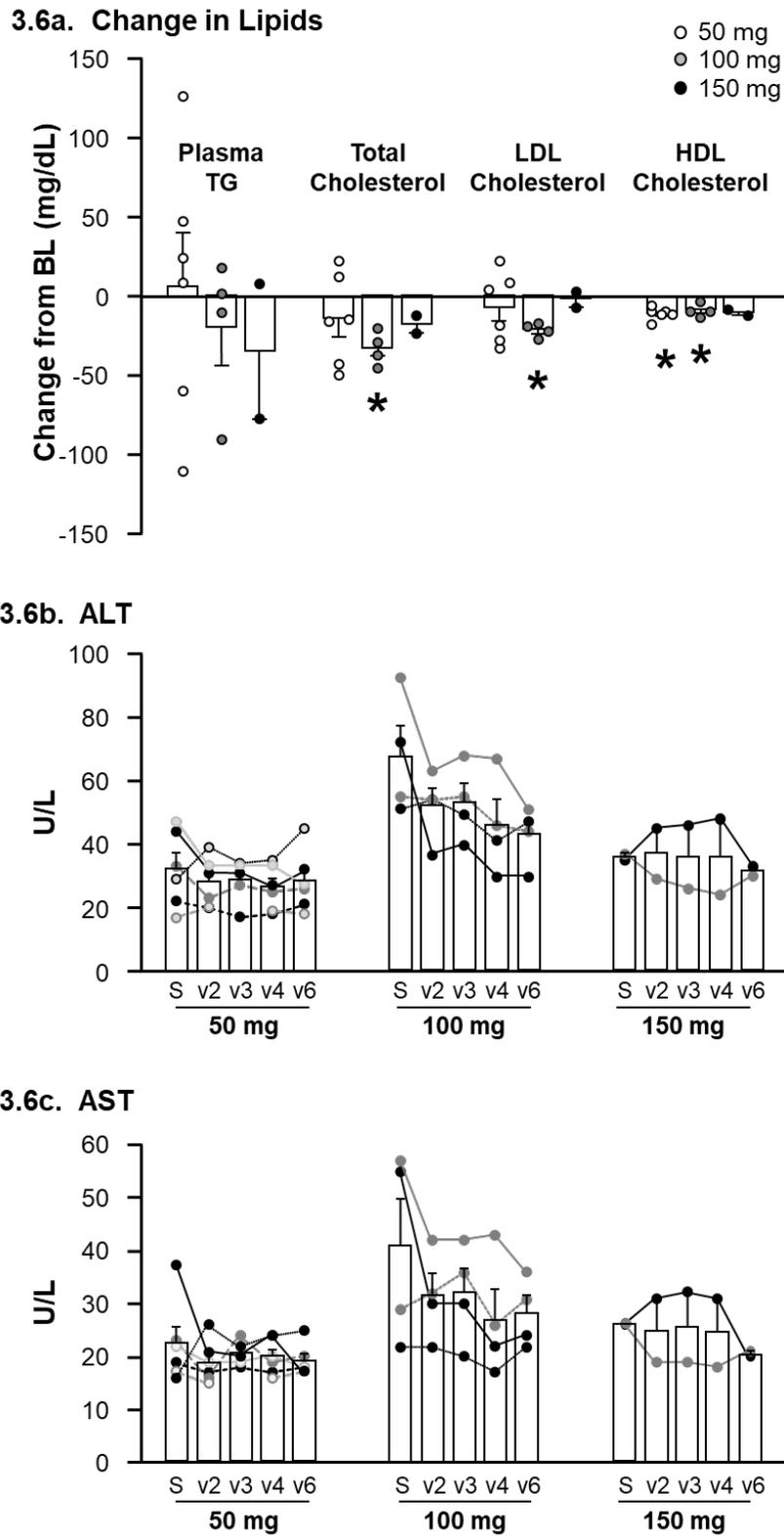
3.5f.



Legend: 3.5a. AUC of the absolute DNL-16:0 before (□) and post-treatment (■). Repeated-measures ANOVA was performed to test the effect of drug on DNL-16:0 before and after the treatment between the three groups. During the

analysis, dose was used as a between-group factor and time was used as within-group factor. Within-group analyses revealed that for all subjects combined, DNL-16:0 was reduced significantly ($P<0.001$). The reduction in DNL-16:0 was significantly different within each group ($P<0.001$). Post hoc analysis revealed that the reduction in DNL-16:0 was significant in all three groups (50 mg, $P=0.027$, 100 mg, $P=0.007$ and 150 mg group, $P<0.001$). **3.5b.** Liver fat measured by MRI scan (PDFF). Liver fat was calculated for a selected region of interest (ROI). Repeated measure ANOVA was performed to test the effect of drug on liver fat measured via MRI scan (IHTG) over time. During the analysis, dose was used as a between groups factor and time was used as within groups factor. Within group analysis revealed that combined all subjects, IHTG was reduced significantly ($P=0.048$). The trend of decrease in IHTG observed within each group was not different i.e., different doses of the drug reduced IHTG in a similar pattern ($P=0.732$). Post hoc analysis revealed that this effect was primarily driven by 100 mg group ($P=0.06$). IHTG tended to reduce in 50 mg group ($P=0.137$) but not in 100 mg group ($P=0.545$). **3.5c.** Liver fat measured by FibroscanTM (CAP, dB/m). Repeated measure ANOVA was performed to test the effect of drug on liver fat measured via FibroScan (CAP) over time. During the analysis, dose was used as a between groups factor and time was used as within groups factor. Within group analysis revealed that combined all subjects, CAP score was reduced significantly ($P=0.002$). The decrease in CAP score within each group was significantly different ($P=0.020$). Post hoc analysis revealed that this effect was primarily driven by 100 mg group ($P=0.025$) and 150 mg group ($P=0.004$). CAP score did not change in the 50 mg group ($P=0.968$). **3.5d-e.** Relationship between AUC of the drug and percent change in peak absolute DNL and change in total IHTG measured by MRI. Relationship between change in fractional DNL and IHTG. A Pearson correlation analysis was performed to test the relationship between DNL, concentration of drug, and IHTG. Abbreviations: AUC, area under the curve; DNL, de novo lipogenesis; AUC, area-under-the-curve; IHTG, intrahepatic triacylglycerol; CAP, liver fat score measured by FibroScan (db/m); BL, baseline; and FL, follow up (post-treatment).

Figure 3.6: Changes in liver enzymes, plasma lipids, and liver fat



Legend: Data are mean \pm SE. **3.6a.** Student t-test was performed to test if the effect of drug on the lipid panel. * $P < 0.05$ represents significant difference from baseline and after completion of the treatment. Only changes in lipid panel for each group is shown in the figure. One-way ANOVA was performed to test if the change observed in the lipid panel, was different between three doses. No differences were observed for total cholesterol ($P=0.487$), high density lipoprotein cholesterol ($P=0.551$), low density lipoprotein cholesterol ($P=0.361$), and plasma-TG ($P=0.740$). **3.6b.** Repeated measure ANOVA was performed to test the effect of drug on ALT values over time. During the analysis, dose was used as a between groups factor and time was used as within groups factor. Overall, ALT values reduced significantly ($P=0.038$). However, the reduction in ALT levels were not different between three doses ($P=0.223$). Between groups analysis suggested that ALT values were significantly different between three groups ($P=0.017$). Pairwise comparison analysis (Sidak-Holm post hoc) revealed that only 100 mg group's ALT values were significantly higher compare to 50 mg group ($P=0.018$) but were not different compare to 150 mg group ($P=0.170$). No differences were observed between 50 mg and 100 mg group ($P=0.880$). **3.6c.** Repeated measure ANOVA was performed to test the effect of drug on AST values over time. During the analysis, dose was used as a between groups factor and time was used as within groups factor. Within group analysis revealed that AST values tended to decrease ($P=0.075$). Decrease in AST observed within each group was not different i.e., different doses of drug reduced AST in a similar pattern ($P=0.617$). Between groups analysis suggested that AST values tended to be different between three groups ($P=0.087$). Pairwise comparison analysis (Sidak-Holm post hoc) revealed that only 100 mg group's AST values were higher compare to 50 mg group ($P=0.095$) but were similar to 150 mg group ($P=0.501$). No differences were observed between 50 mg and 100 mg group ($P=0.901$). Abbreviations: ALT, alanine transaminase; AST, aspartate aminotransferase; PDFF, proton density fat fraction; IHTG, intrahepatic triacylglycerols; CAP, units for liver fat measured by FibroScan™; Sc, screening value.

REFERENCES

1. Browning, J. D., & Horton, J. D. (2004). Molecular mediators of hepatic steatosis and liver injury. *J Clin Invest*, *114*(2), 147-152. doi:10.1172/JCI22422. **PMC449757**.
2. Younossi, Z. M., Koenig, A. B., Abdelatif, D., Fazel, Y., Henry, L., & Wymer, M. (2016). Global epidemiology of nonalcoholic fatty liver disease: Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology*, *64*(1), 73-84. doi:10.1002/hep.28431. **PMID: 26707365**.
3. Calzadilla Bertot, L., & Adams, L. A. (2016). The natural course of non-alcoholic fatty liver disease. *Int J Mol Sci*, *17*(5). doi:10.3390/ijms17050774. **PMC4881593**.
4. Ray, K. (2013). NAFLD-the next global epidemic. *Nat Rev Gastroenterol Hepatol*, *10*(11), 621. doi:10.1038/nrgastro.2013.197.
5. Eslam, M., Valenti, L., & Romeo, S. (2018). Genetics and epigenetics of NAFLD and NASH: Clinical impact. *J Hepatol*, *68*(2), 268-279. doi:10.1016/j.jhep.2017.09.003. **PMID: 29122391**.
6. Lambert, J. E., Ramos-Roman, M. A., Browning, J. D., & Parks, E. J. (2014). Increased de novo lipogenesis is a distinct characteristic of individuals with nonalcoholic fatty liver disease. *Gastroenterology*, *146*(3), 726-735. doi:10.1053/j.gastro.2013.11.049. **PMC6276362**.
7. Donnelly, K. L., Smith, C. I., Schwarzenberg, S. J., Jessurun, J., Boldt, M. D., & Parks, E. J. (2005). Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J Clin Invest*, *115*(5), 1343-1351. doi:10.1172/JCI23621. **PMC1087172**.
8. Postic, C., & Girard, J. (2008). Contribution of de novo fatty acid synthesis to hepatic steatosis and insulin resistance: lessons from genetically engineered mice. *J Clin Invest*, *118*(3), 829-838. doi:10.1172/JCI34275. **PMC2254980**.
9. Ahmed, M. H., & Byrne, C. D. (2007). Modulation of sterol regulatory element binding proteins (SREBPs) as potential treatments for non-alcoholic fatty liver disease (NAFLD). *Drug Discov Today*, *12*(17-18), 740-747. doi:10.1016/j.drudis.2007.07.009. **PMID: 17826687**.
10. Byrne, C. D., Olufadi, R., Bruce, K. D., Cagampang, F. R., & Ahmed, M. H. (2009). Metabolic disturbances in non-alcoholic fatty liver disease. *Clin Sci (Lond)*, *116*(7), 539-564. doi:10.1042/CS20080253. **PMID: 19243311**.
11. Finck, B. N. (2018). Targeting metabolism, insulin resistance, and diabetes to treat nonalcoholic steatohepatitis. *Diabetes*, *67*(12), 2485-2493. doi:10.2337/dbi18-0024. **PMC6245219**.
12. Imai, N., & Cohen, D. E. (2018). Trimming the fat: Acetyl-CoA Carboxylase inhibition for the management of NAFLD. *Hepatology*, *68*(6), 2062-2065. doi:10.1002/hep.30206. **PMID: 30076622**.
13. Kim, C. W., Addy, C., Kusunoki, J., Anderson, N. N., Deja, S., Fu, X., Burgess, S. C., Li, C., Ruddy, M., Chakravarthy, M., Previs, S., Milstein, S., Fitzgerald, K., Kelley, D. E., & Horton, J. D. (2017). Acetyl CoA

Carboxylase inhibition reduces hepatic steatosis but elevates plasma triglycerides in mice and humans: A bedside to bench investigation. *Cell Metab*, 26(2), 394-406 e396. doi:10.1016/j.cmet.2017.07.009.

PMC5603267.

14. Savage, D. B., Choi, C. S., Samuel, V. T., Liu, Z. X., Zhang, D., Wang, A., Zhang, X. M., Cline, G. W., Yu, X. X., Geisler, J. G., Bhanot, S., Monia, B. P., & Shulman, G. I. (2006). Reversal of diet-induced hepatic steatosis and hepatic insulin resistance by antisense oligonucleotide inhibitors of acetyl-CoA carboxylases 1 and 2. *J Clin Invest*, 116(3), 817-824. doi:10.1172/JCI27300. **PMC1366503.**
15. Chakravarthy, M. V., Pan, Z., Zhu, Y., Tordjman, K., Schneider, J. G., Coleman, T., Turk, J., & Semenkovich, C. F. (2005). "New" hepatic fat activates PPARalpha to maintain glucose, lipid, and cholesterol homeostasis. *Cell Metab*, 1(5), 309-322. doi:10.1016/j.cmet.2005.04.002. **PMID: 16054078.**
16. Ntambi, J. M., Miyazaki, M., Stoehr, J. P., Lan, H., Kendzioriski, C. M., Yandell, B. S., Song, Y., Cohen, P., Friedman, J. M., & Attie, A. D. (2002). Loss of stearoyl-CoA desaturase-1 function protects mice against adiposity. *Proc Natl Acad Sci U S A*, 99(17), 11482-11486. doi:10.1073/pnas.132384699. **PMC123282.**
17. Neschen, S., Morino, K., Hammond, L. E., Zhang, D., Liu, Z. X., Romanelli, A. J., Cline, G. W., Pongratz, R. L., Zhang, X. M., Choi, C. S., Coleman, R. A., & Shulman, G. I. (2005). Prevention of hepatic steatosis and hepatic insulin resistance in mitochondrial acyl-CoA:glycerol-sn-3-phosphate acyltransferase 1 knockout mice. *Cell Metab*, 2(1), 55-65. doi:10.1016/j.cmet.2005.06.006. **PMID: 16054099.**
18. Yamaguchi, K., Yang, L., McCall, S., Huang, J., Yu, X. X., Pandey, S. K., Bhanot, S., Monia, B. P., Li, Y. X., & Diehl, A. M. (2007). Inhibiting triglyceride synthesis improves hepatic steatosis but exacerbates liver damage and fibrosis in obese mice with nonalcoholic steatohepatitis. *Hepatology*, 45(6), 1366-1374. doi:10.1002/hep.21655. **PMID: 17476695.**
19. Loomba, R., Kayali, Z., Nouredin, M., Ruane, P., Lawitz, E. J., Bennett, M., Wang, L., Harting, E., Tarrant, J. M., McColgan, B. J., Chung, C., Ray, A. S., Subramanian, G. M., Myers, R. P., Middleton, M. S., Lai, M., Charlton, M., & Harrison, S. A. (2018). GS-0976 reduces hepatic steatosis and fibrosis markers in patients with nonalcoholic fatty liver disease. *Gastroenterology*, 155(5), 1463-1473 e1466. doi:10.1053/j.gastro.2018.07.027. **PMID: 30059671.**
20. Lawitz, E. J., Coste, A., Poordad, F., Alkhoury, N., Loo, N., McColgan, B. J., Tarrant, J. M., Nguyen, T., Han, L., Chung, C., Ray, A. S., McHutchison, J. G., Subramanian, G. M., Myers, R. P., Middleton, M. S., Sirlin, C., Loomba, R., Nyangau, E., Fitch, M., Li, K., & Hellerstein, M. (2018). Acetyl-CoA Carboxylase inhibitor GS-0976 for 12 weeks reduces hepatic de novo lipogenesis and steatosis in patients with nonalcoholic

- steatohepatitis. *Clin Gastroenterol Hepatol*, 16(12), 1983-1991 e1983. doi:10.1016/j.cgh.2018.04.042. **PMID: 29705265.**
21. Stiede, K., Miao, W., Blanchette, H. S., Beysen, C., Harriman, G., Harwood, H. J., Jr., Kelley, H., Kapeller, R., Schmalbach, T., & Westlin, W. F. (2017). Acetyl-coenzyme A carboxylase inhibition reduces de novo lipogenesis in overweight male subjects: A randomized, double-blind, crossover study. *Hepatology*, 66(2), 324-334. doi:10.1002/hep.29246. **PMID: 29705265.**
 22. Wu, M., Singh, S. B., Wang, J., Chung, C. C., Salituro, G., Karanam, B. V., Lee, S. H., Powles, M., Ellsworth, K. P., Lassman, M. E., Miller, C., Myers, R. W., Tota, M. R., Zhang, B. B., & Li, C. (2011). Antidiabetic and antisteatotic effects of the selective fatty acid synthase (FAS) inhibitor platensimycin in mouse models of diabetes. *Proc Natl Acad Sci U S A*, 108(13), 5378-5383. doi:10.1073/pnas.1002588108. **PMID: 21511966.**
 23. Jones, S. F., & Infante, J. R. (2015). Molecular Pathways: Fatty Acid Synthase. *Clin Cancer Res*, 21(24), 5434-5438. doi:10.1158/1078-0432.CCR-15-0126. **PMID: 26519059.**
 24. Buckley, D., Duke, G., Heuer, T. S., O'Farrell, M., Wagman, A. S., McCulloch, W., & Kemble, G. (2017). Fatty acid synthase - Modern tumor cell biology insights into a classical oncology target. *Pharmacol Ther*, 177, 23-31. doi:10.1016/j.pharmthera.2017.02.021. **PMID: 28202364.**
 25. Duke, G., Wagman, A., Buckley, D., McCulloch, W., Feigh, M., Veidal, S. S., & Kemble, G. (2017). Fatty acid synthase inhibitor TVB-3664 reduces collagen accumulation in bleomycin-induced murine skin fibrosis and reverses multiple components of diet-induced and biopsy-confirmed nonalcoholic steatohepatitis in mice treated with or without co-administered pirfenidone. *Hepatology*, 66(S1), 1056A.
 26. Parks, E. J., Manrique, C. M., Syed-Abdul, M. M., Gaballah, A. H., Hammoud, G. M., Buckley, D., Duke, G., McCulloch, W., & Kemble, G. (2017). Pharmacologic inhibition of FASN reverses diet-induced steatohepatitis in mice and inhibits lipogenesis in humans. *Hepatology*, 66(S1), 1045A.
 27. Brenner, A., Infante, J., Patel, M., Arkenau, H.-T., Voskoboynik, M., Borazanci, E., Falchook, G., Molife, L. R., Pant, S., Dean, E., Pelosof, L., Jones, S., Rubino, C., McCulloch, W., Zhukova-Harrill, V., Kemble, G., O'Farrell, M., & Burris, H. A. (2015). Abstract A54: First-in-human study of the first-in-class fatty acid synthase (FASN) inhibitor, TVB-2640 as monotherapy or in combination - final results of dose escalation. *Molecular Cancer Therapeutics*, 14(12 Supplement 2), A54-A54. doi:10.1158/1535-7163.targ-15-a54.
 28. Duke, G., Wagman, A. S., Buckley, D., McCulloch, W., & Kemble, G. (2017). Establishing the foundation for a novel, first-in-class, fatty acid synthase inhibitor, TVB-2640, for the treatment of NASH. *Journal of Hepatology*, 66(1), S99-S100. doi:10.1016/S0168-8278(17)30460-9.
 29. Grundy, S. M., Brewer, H. B., Jr., Cleeman, J. I., Smith, S. C., Jr., Lenfant, C., American Heart, A., National Heart, L., & Blood, I. (2004). Definition

- of metabolic syndrome: Report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition. *Circulation*, 109(3), 433-438. doi:10.1161/01.CIR.0000111245.75752.C6. **PMID: 14744958.**
30. Ahren, B., & Larsson, H. (2002). Quantification of insulin secretion in relation to insulin sensitivity in nondiabetic postmenopausal women. *Diabetes*, 51 Suppl 1, S202-211.
 31. Kannengiesser, S., Neji, R., & Zhong, X. (2014). Case Study LiverLab. *MAGNETOM Flash*, 3(58), 18-19.
 32. Chavez-Jauregui, R. N., Mattes, R. D., & Parks, E. J. (2010). Dynamics of fat absorption and effect of sham feeding on postprandial lipemia. *Gastroenterology*, 139(5), 1538-1548. doi:10.1053/j.gastro.2010.05.002. **PMC2948783.**
 33. Hawkins, M., Gabriely, I., Wozniak, R., Vilcu, C., Shamoon, H., & Rossetti, L. (2002). Fructose improves the ability of hyperglycemia per se to regulate glucose production in type 2 diabetes. *Diabetes*, 51(3), 606-614. doi:10.2337/diabetes.51.3.606. **PMID: 11872657.**
 34. Parks, E. J., Skokan, L. E., Timlin, M. T., & Dingfelder, C. S. (2008). Dietary sugars stimulate fatty acid synthesis in adults. *J Nutr*, 138(6), 1039-1046. doi:10.1093/jn/138.6.1039. **PMC2546703.**
 35. Parks, E. J., Krauss, R. M., Christiansen, M. P., Neese, R. A., & Hellerstein, M. K. (1999). Effects of a low-fat, high-carbohydrate diet on VLDL-triglyceride assembly, production, and clearance. *J Clin Invest*, 104(8), 1087-1096. doi:10.1172/JCI6572. **PMC408572.**
 36. Barrows, B. R., & Parks, E. J. (2006). Contributions of different fatty acid sources to very low-density lipoprotein-triacylglycerol in the fasted and fed states. *J Clin Endocrinol Metab*, 91(4), 1446-1452. doi:10.1210/jc.2005-1709. **PMID: 16449340.**
 37. Hellerstein, M. K., & Neese, R. A. (1999). Mass isotopomer distribution analysis at eight years: theoretical, analytic, and experimental considerations. *Am J Physiol*, 276(6 Pt 1), E1146-1170. **PMID: 10362629.**
 38. Santoro, N., Caprio, S., Pierpont, B., Van Name, M., Savoye, M., & Parks, E. J. (2015). Hepatic de novo lipogenesis in obese youth is modulated by a common variant in the GCKR gene. *J Clin Endocrinol Metab*, 100(8), E1125-1132. doi:10.1210/jc.2015-1587. **PMC4524990.**
 39. Chong, M. F., Fielding, B. A., & Frayn, K. N. (2007). Mechanisms for the acute effect of fructose on postprandial lipemia. *Am J Clin Nutr*, 85(6), 1511-1520. doi:10.1093/ajcn/85.6.1511. **PMID: 17556686.**
 40. Aarsland, A., Chinkes, D., & Wolfe, R. R. (1997). Hepatic and whole-body fat synthesis in humans during carbohydrate overfeeding. *Am J Clin Nutr*, 65(6), 1774-1782. doi:10.1093/ajcn/65.6.1774. **PMID: 9174472.**
 41. Hudgins, L. C., Parker, T. S., Levine, D. M., & Hellerstein, M. K. (2011). A dual sugar challenge test for lipogenic sensitivity to dietary fructose. *J Clin Endocrinol Metab*, 96(3), 861-868. doi:10.1210/jc.2010-2007. **PMC3047222.**

42. O'Farrell, M., Crowley, R., Heuer, T., Fridlib, M., Buckley, D., McCulloch, W., & Kemble, G. (2015). Biomarker analyses from dose escalation phase of FASN inhibitor TVB-2640 phase 1 study shows target engagement in solid tumor patients. *AACR; Molecular Cancer Therapeutics*, 14(12). doi:DOI: 10.1158/1535-7163.TARG-15-B13.
43. Pappas, A., Johnsen, S., Liu, J. C., & Eisinger, M. (2009). Sebum analysis of individuals with and without acne. *Dermatoendocrinol*, 1(3), 157-161. doi:10.4161/derm.1.3.8473. **PMC2835908**.
44. Esler, W. P., Tesz, G. J., Hellerstein, M. K., Beysen, C., Sivamani, R., Turner, S. M., Watkins, S. M., Amor, P. A., Carvajal-Gonzalez, S., Geoly, F. J., Biddle, K. E., Purkal, J. J., Fitch, M., Buckeridge, C., Silvia, A. M., Griffith, D. A., Gorgoglione, M., Hassoun, L., Bosanac, S. S., Vera, N. B., Rolph, T. P., Pfefferkorn, J. A., & Sonnenberg, G. E. (2019). Human sebum requires de novo lipogenesis, which is increased in acne vulgaris and suppressed by acetyl-CoA carboxylase inhibition. *Sci Transl Med*, 11(492). doi:10.1126/scitranslmed.aau8465. **PMID: 31092695**.
45. Funai, K., Song, H., Yin, L., Lodhi, I. J., Wei, X., Yoshino, J., Coleman, T., & Semenkovich, C. F. (2013). Muscle lipogenesis balances insulin sensitivity and strength through calcium signaling. *J Clin Invest*, 123(3), 1229-1240. doi:10.1172/JCI65726. **PMC3582136**.
46. Goedeke, L., Bates, J., Vatner, D. F., Perry, R. J., Wang, T., Ramirez, R., Li, L., Ellis, M. W., Zhang, D., Wong, K. E., Beysen, C., Cline, G. W., Ray, A. S., & Shulman, G. I. (2018). Acetyl-CoA Carboxylase inhibition reverses NAFLD and hepatic insulin resistance but promotes hypertriglyceridemia in rodents. *Hepatology*, 68(6), 2197-2211. doi:10.1002/hep.30097. **PMC6251774**.
47. Razani, B., Zhang, H., Schulze, P. C., Schilling, J. D., Verbsky, J., Lodhi, I. J., Topkara, V. K., Feng, C., Coleman, T., Kovacs, A., Kelly, D. P., Saffitz, J. E., Dorn, G. W., 2nd, Nichols, C. G., & Semenkovich, C. F. (2011). Fatty acid synthase modulates homeostatic responses to myocardial stress. *J Biol Chem*, 286(35), 30949-30961. doi:10.1074/jbc.M111.230508. **PMC3162454**.
48. Lee, S., Zhang, C., Liu, Z., Klevstig, M., Mukhopadhyay, B., Bergentall, M., Cinar, R., Stahlman, M., Sikanic, N., Park, J. K., Deshmukh, S., Harzandi, A. M., Kuijpers, T., Grotli, M., Elsasser, S. J., Piening, B. D., Snyder, M., Smith, U., Nielsen, J., Backhed, F., Kunos, G., Uhlen, M., Boren, J., & Mardinoglu, A. (2017). Network analyses identify liver-specific targets for treating liver diseases. *Mol Syst Biol*, 13(8), 938. doi:10.15252/msb.20177703. **PMC5572395**.
49. Roth, E. M., & Davidson, M. H. (2018). PCSK9 Inhibitors: Mechanism of action, efficacy, and safety. *Rev Cardiovasc Med*, 19(S1), S31-S46. **PMID: 30207556**.

SUPPORTING INFORMATION

First-in-class fatty acid synthase inhibitor TVB-2640 reduces hepatic de novo lipogenesis in males with metabolic abnormalities

Majid M. Syed-Abdul¹, Elizabeth J. Parks^{1,2}, Ayman H. Gaballah³, Kimberlee Bingham¹, Ghassan M. Hammoud², George Kemble⁴, Douglas Buckley⁴, William McCulloch⁴, Camila M. Manrique⁵

¹Department of Nutrition and Exercise Physiology, ²Department of Medicine-Division of Gastroenterology and Hepatology, ³Department of Radiology, and , and University of Missouri School of Medicine, Columbia, MO; ⁴3-V Biosciences, Inc., Menlo Park, CA ⁵Department of Medicine-Division of Endocrinology, University of Missouri School of Medicine, Columbia, MO

Camila Manrique Acevedo, MD, manriquec@health.missouri.edu
Elizabeth J. Parks, PhD, parksej@missouri.edu

Supplementary Tables S3.1 - S3.3
Figures S3.1 - S3.4

Supplementary Table S3.1. Pre-study diet and study diet

Energy	Pre-study diet	Study diet	P-value
Total energy (kcal)	3194 ± 315	3213 ± 168	0.948
Carbohydrates (g)	354 ± 45	386 ± 22	0.404
Total sugars (g)	151 ± 25	163 ± 14	0.546
Fiber (g)	20 ± 3	25 ± 2	0.133
Dietary fat (g)	145 ± 15	130 ± 7	0.312
MUFA (g)	51 ± 6	46 ± 2	0.330
PUFA (g)	31 ± 4	24 ± 1	0.077
Saturated fat (g)	49 ± 5	48 ± 3	0.809
Cholesterol (mg)	535 ± 68	468 ± 30	0.339
Proteins (g)	121 ± 11	137 ± 6	0.166
Sodium (mg)	5618 ± 662	5092 ± 363	0.372
Potassium (mg)	3369 ± 372	3861 ± 137	0.221

Legend: Data are mean ± SE. Analysis is by paired t-test.

Supplementary Table S3.2. Blood pressure measurements during the study

Systolic BP	50 mg	100 mg	150 mg	Time X group¹
Baseline	136 ± 6	135 ± 4	122 ± 15	0.413
2d	143 ± 5	130 ± 7	126 ± 8	
5d	131 ± 4	133 ± 6	122 ± 5	
7d	132 ± 5	129 ± 10	119 ± 7	
post-treatment	131 ± 5	135 ± 9	130 ± 5	
6d follow-up	131 ± 3	132 ± 7	125 ± 7	
² Within group ANOVA	0.114	0.860	0.860	
Diastolic BP	50 mg	100 mg	150 mg	Time X group¹
Baseline	84 ± 5	93 ± 7	77 ± 2	0.386
2d	81 ± 3	81 ± 5	82 ± 3	
5d	79 ± 3	80 ± 4	79 ± 2	
7d	80 ± 5	75 ± 4	71 ± 3	
post-treatment	81 ± 2	82 ± 5	83 ± 10	
6d follow-up	80 ± 3	80 ± 3	74 ± 2	
² Within group ANOVA	0.838	0.308	0.267	

Legend: Data are mean ± SE. Two-way repeated measure ANOVA was performed to test the effects of treatment within each group and differences between groups. Within-subject effects suggested no change in systolic blood pressure (ANOVA, $P=0.413$) but diastolic blood pressure changed (ANOVA, $P=0.045$). ¹ No time x group interactions (between group effects) were found by ANOVA. ² Within each group, the P -value reflects the effect of time.

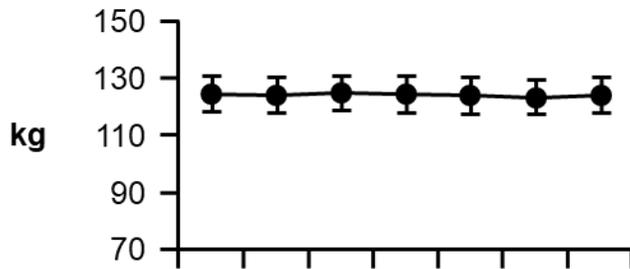
Supplementary Table S3.3. Adverse drug reaction monitoring over time

Adverse Drug Reactions	50 mg			100 mg			150 mg		
	During study	Safety visit 6	Post safety visit 6	During study	Safety visit 6	Post safety visit 6	During study	Safety visit 6	Post safety visit 6
Constipation	-	mild (1)	-	-	-	-	-	-	-
Diarrhea	-	-	-	mild (11)	-	-	-	-	-
Disturbed bowel habits	-	-	-	mild (10)	-	-	-	-	-
Dry throat	-	-	-	-	-	-	mild (8)	-	-
Dry skin	mild (6)	-	-	-	-	-	-	-	-
Fatigue	-	-	-	-	-	-	-	-	-
Hair loss	-	-	-	-	-	moderate (9)	-	moderate (8)	-
Headache	-	-	-	mild (12)	-	-	-	-	-
Loss of cravings	mild (3)	-	-	-	-	-	-	-	-
Peeling skin on fingertips	-	-	-	-	-	mild (9)	-	-	-

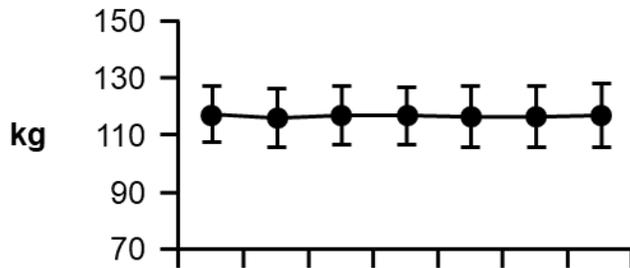
Legend: The numbers in the parenthesis are the subject ID# 1-12. Reporting of adverse drug reactions occurred in person, at each visit during the study, and one week after completion of drug treatment (indicated here as safety visit 6). Subjects were asked to also contact study staff within 2 weeks after the final safety visit 6, if they noticed any side effects. Severity of adverse effects were rated as mild, moderate, or severe. No severe effects were reported in this study. For the subjects who already exhibited adverse effects (listed above) before starting the drug treatment, these adverse effects are reported only if they were worsened during and/or after completion of the study.

Supplementary Figure S3.1. Subject body weights during the study

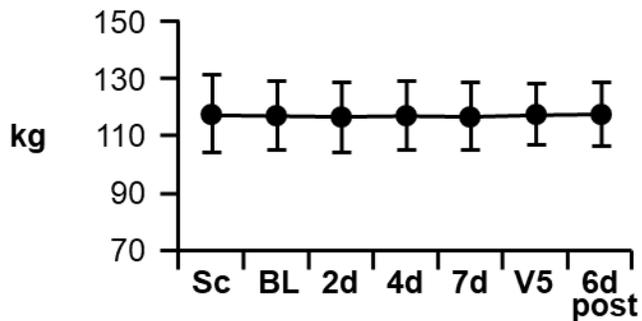
S3.1a. 50 mg group



S3.1b. 100 mg group

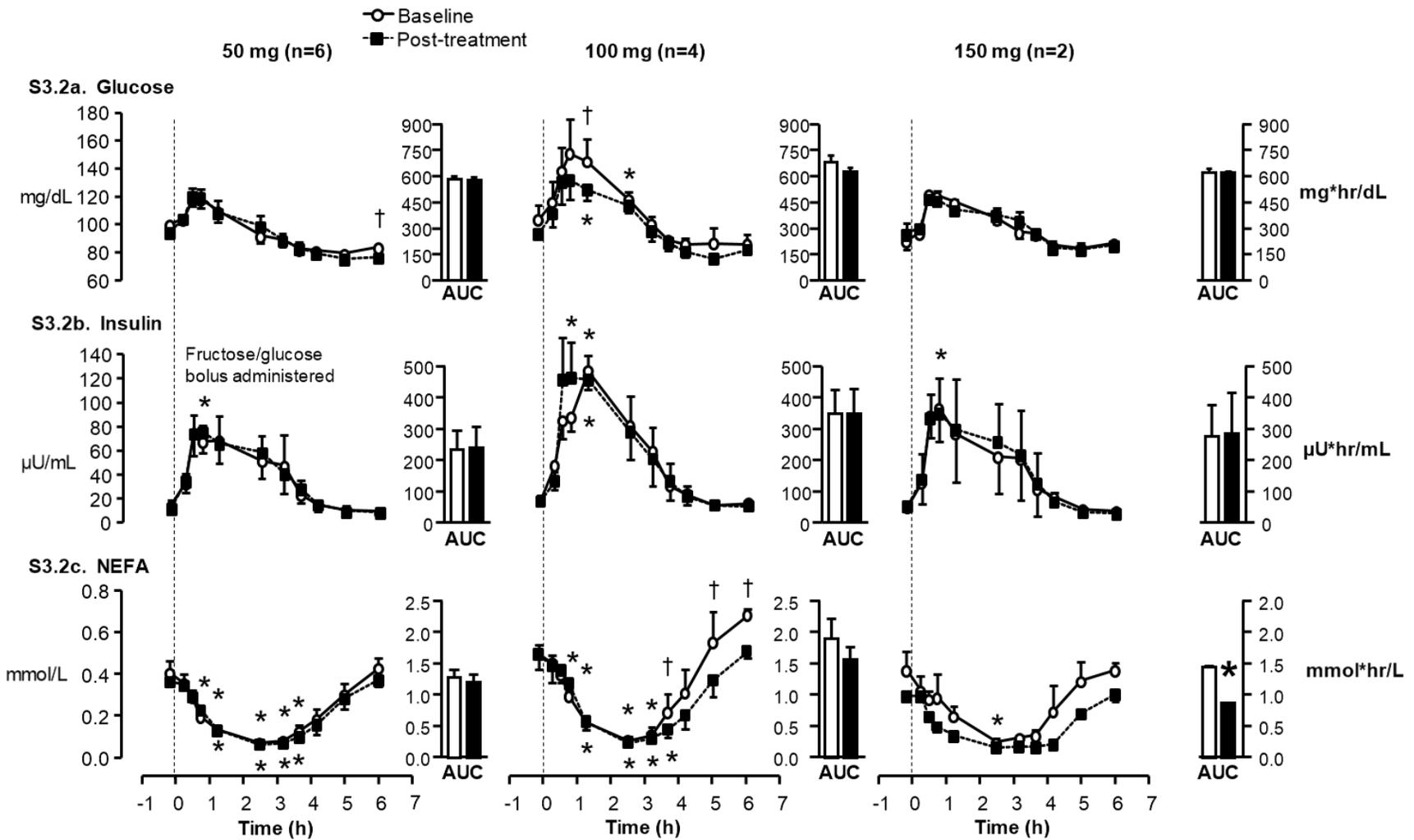


S3.1c. 150 mg group



Legend: S3.1a-c. Data are mean \pm SE. Repeated measure ANOVA was performed to test the change in body weight during the study between three groups. During the analysis, dose was used as a between groups factor and time was used as within groups factor. Body weight remained stable during the study for all subjects ($P=0.305$). No post hoc analysis was performed.

Supplementary Figure S3.2. Changes in plasma insulin, glucose, and NEFA concentrations

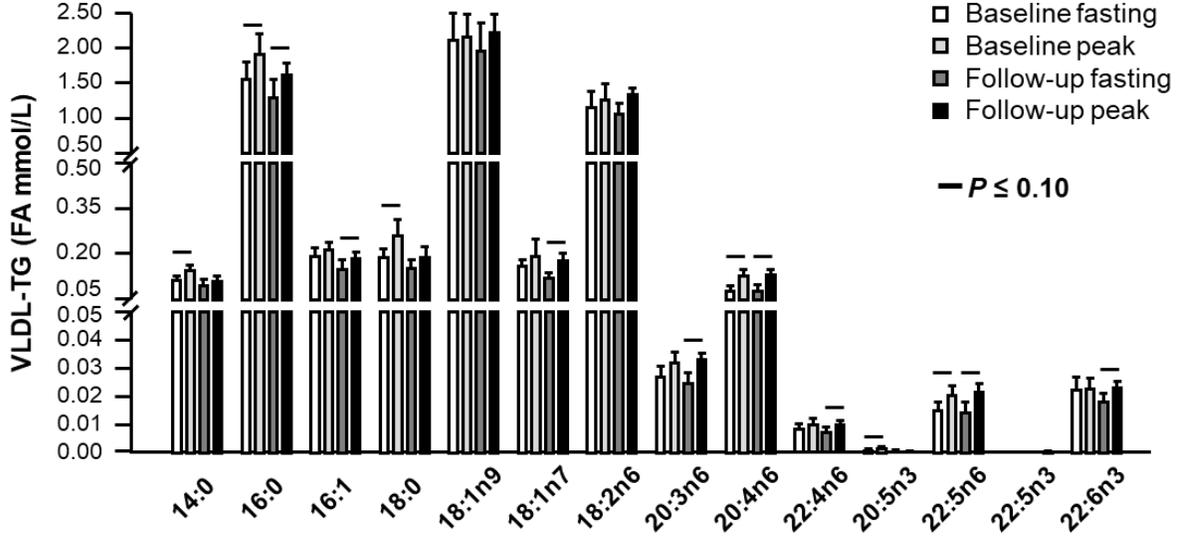


Legend: Data are mean ± SE. Repeated-measures ANOVA was conducted using two within factors and one between factor. Time post consumption of fructose/glucose bolus was used as a first factor, 10 days of treatment was used as a

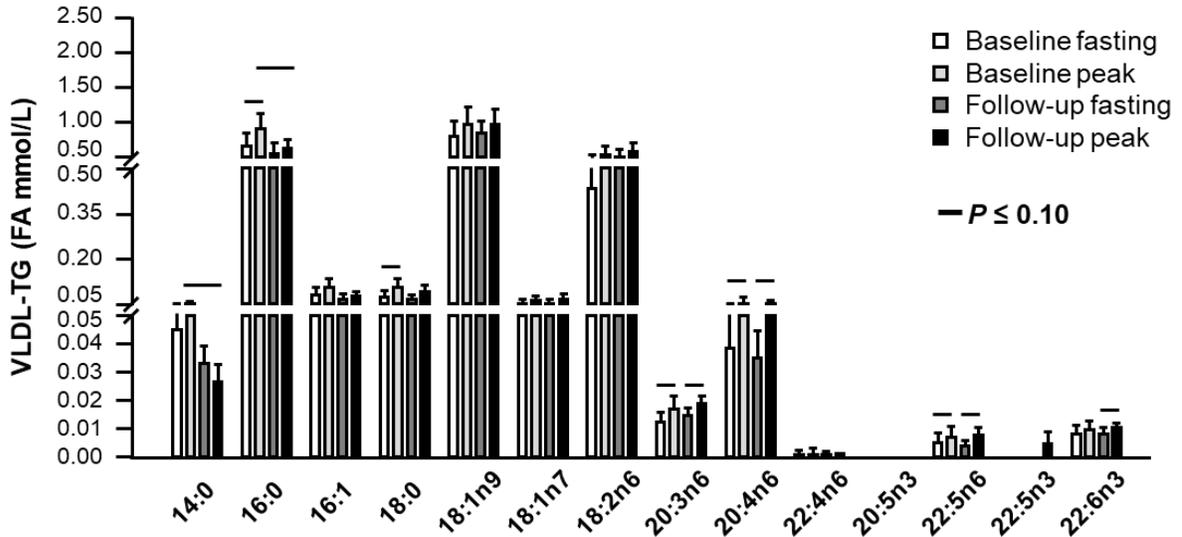
second factor for within factors, and group (dose) was used as a between factor. **S3.2a.** Plasma glucose concentration in response to fructose/glucose tolerance test. For between factor analysis, plasma glucose did not change for all subjects ($P=0.222$) or between groups ($P=0.185$). Within factor analysis revealed that plasma glucose did not change in response to fructose/glucose bolus for all three groups before (50 mg, $P=0.652$; 100 mg, $P=0.742$; 150 mg, $P=0.523$) and in two groups after the drug treatment (50 mg, $P=0.273$; 100 mg $P=0.050$; 150 mg, $P=0.398$). **S3.2b.** Plasma insulin concentration in response to fructose/glucose tolerance test. For between factor analysis, plasma insulin did not change for all subjects ($P=0.424$) or between groups ($P=0.845$). Within factor analysis revealed that plasma insulin did not change in response to fructose/glucose bolus for all three groups before (50 mg, $P=0.392$; 100 mg, $P=0.277$; 150 mg, $P=0.658$) and after the drug treatment (50 mg, $P=0.248$; 100 mg $P=0.141$; 150 mg, $P=0.463$). **S3.2c.** Plasma NEFA concentration in response to fructose/glucose tolerance test. For between factor analysis, plasma NEFA values were significantly lower for all subjects ($P=0.036$) however, it was not different between groups ($P=0.335$). Within factor analysis revealed that plasma NEFA did not change in response to fructose/glucose bolus for all three groups before (50 mg, $P=0.212$; 100 mg, $P=0.075$; 150 mg, $P=0.106$) and after the drug treatment (50 mg, $P=0.059$; 100 mg $P=0.076$; 150 mg, $P=0.187$). * $P\leq 0.05$ compare to fasting value. † represents significant difference between baseline and post-treatment at each point ($P\leq 0.05$). Open circles (○) represent baseline values (before treatment) and closed squares (■) are post-treatment values. AUC values are presented as bar graph next to each graph. Open bars (□) represent before treatment and closed bars (■) represent post-treatment. Abbreviations: NEFA, nonesterified fatty acids; AUC, area under the curve. * $P\leq 0.05$ for difference between BL and post-treatment at a single time point or between AUCs.

Supplementary Figure S3.3. Changes in the fatty acid composition of fasting and peak VLDL-TG

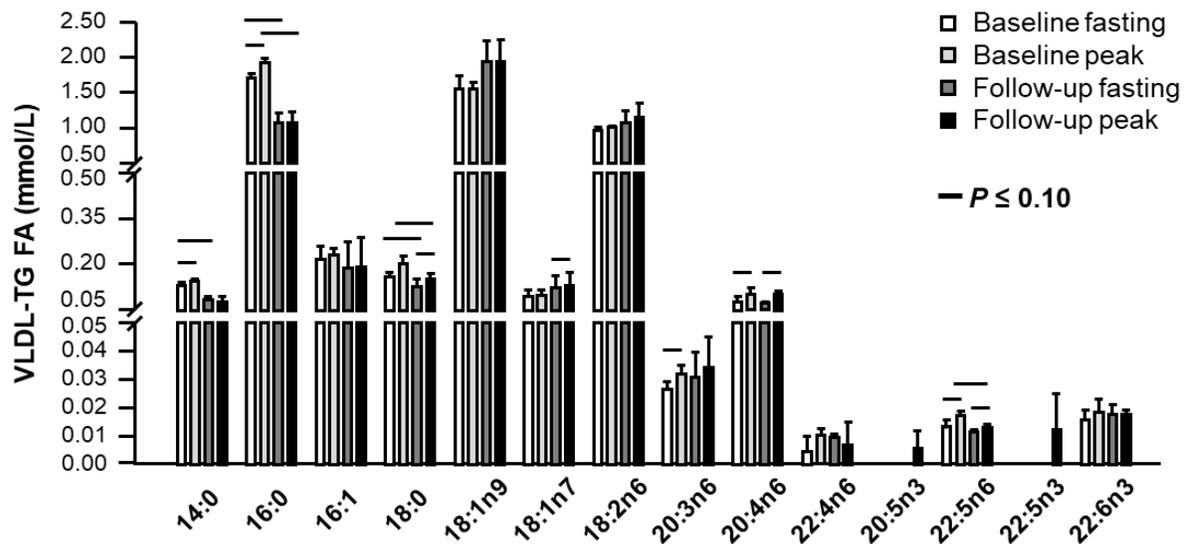
S3.3a. 50 mg group



S3.3b. 100 mg group

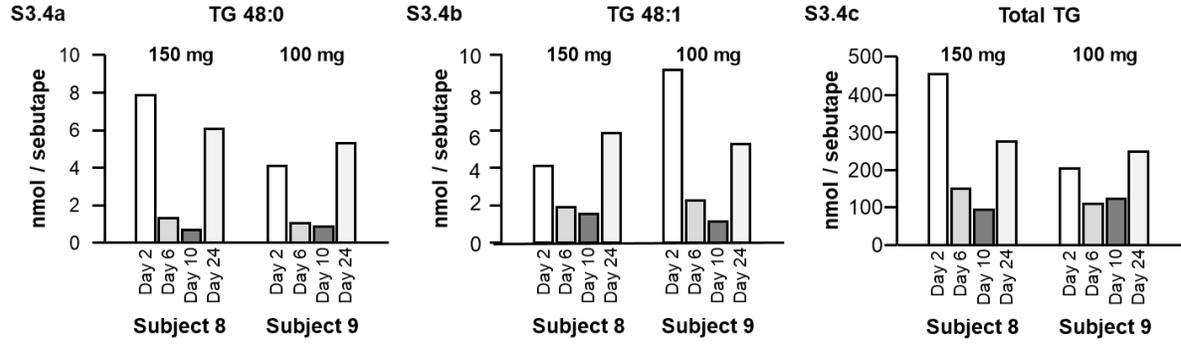


S3.3c. 150 mg group



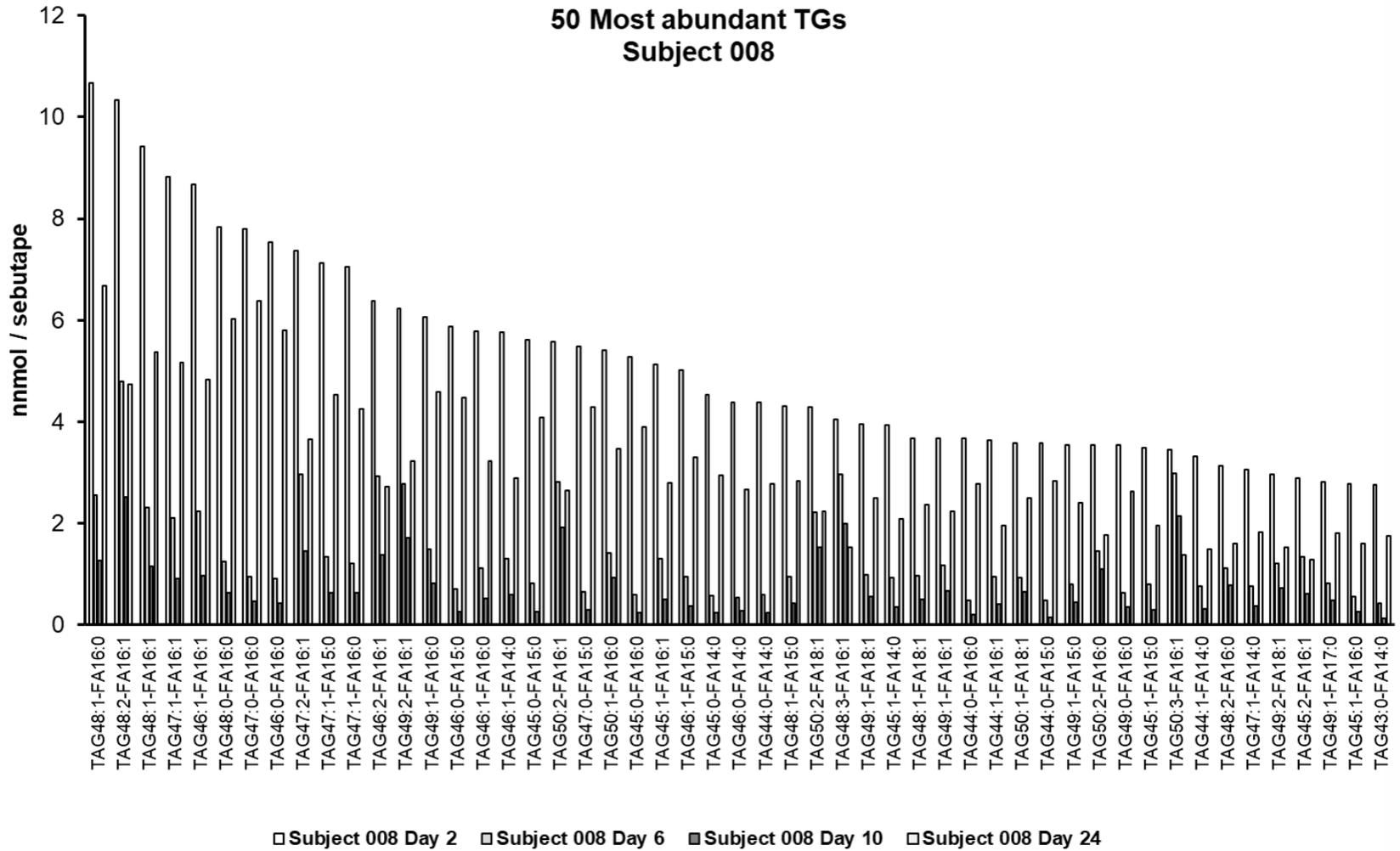
Legend: S3a-c. Data are mean \pm SE for subjects in **S3.3a**, 50 mg group (n=6), **S3.3b**, 100 mg group (n=4), and **S3.3c**, 150 mg group (n=2). Fatty acid (FA) units are those in VLDL-TG, mmol per liter plasma. The bars represent significant differences between values, $P \leq 0.10$. Students t-test was performed to test the difference between fasting and peak values, and baseline and follow-up.

Supplementary Figure S3.4. Changes in selected triacylglycerol concentrations in sebocytes from two subjects



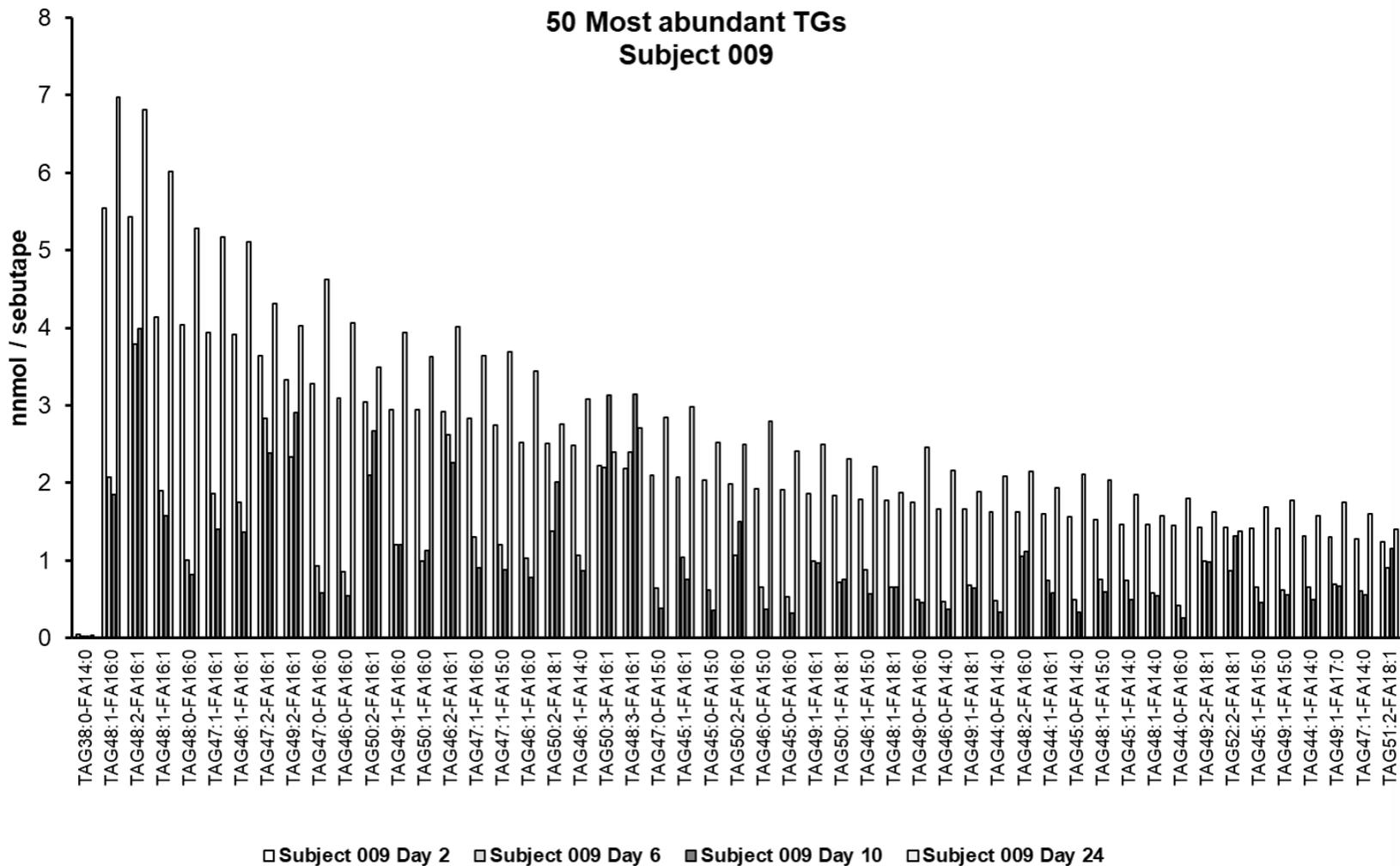
S3.4d

240



S3.4e

241



Legend: Two subjects (#8 and #9) exhibited alopecia associated with drug treatment, which resolved within 4 weeks of termination of treatment. No statistical analyses were performed. **S3.4a-b.** Measurement of sebutape TG 48:0 and TG 48:1 for subject #8 and #9. **3.4c.** Measurement of average of all sebutape TG for subject #8 and #9. **S3.4d-e.** Sebutape analysis for top 50 TG species for subject #8 (**S3.4d**) and #9 (**S3.4e**).

**Chapter IV - The Tailgate Study: Differing metabolic effects of a bout
of excessive eating and drinking**

ABSTRACT

Introduction: Excess energy intake by spectators at a sporting event (i.e., a tailgate) might cause acute negative health effects. However, limited data exist regarding the effects of overeating and alcohol consumption on lipid metabolism and the potential to gain intrahepatic triacylglycerols (IHTG). We tested the hypothesis that overconsumption of food and alcohol would significantly increase both hepatic de novo lipogenesis (DNL) and IHTG. **Methods:** Eighteen males (mean±SD, age: 31.4±7.3 y, BMI: 32.1±5.9 kg/m²) were fed alcoholic drinks to elevate blood alcohol for 5hr, while highly-palatable food was presented. Blood samples were collected and DNL in TG-rich lipoproteins (TRL) was measured by GC/MS, IHTG was measured via MRS (n=15), and substrate oxidation via indirect calorimetry. **Results:** Subjects consumed 5,087±149 kcal (191±25% excess of total daily energy needs including 171±24g alcohol), which increased plasma insulin, glucose, TG, and decreased NEFA (ANOVA $P\leq 0.003$ for all). Both DNL and TRL-TG increased ($P<0.001$) while IHTG did not change in the group as a whole ($P=0.229$). Individual subject data revealed remarkably differing responses for IHTG (9 increased, 5 decreased, 1 did not change). Despite maintaining equal breath alcohol levels, subjects with IHTG elevations exhibited higher DNL, consumed 90% less alcohol ($P=0.048$), tended to consume more carbohydrates, and exhibited lower whole-body fat oxidation (not significant) compared to those whose IHTG was reduced. **Discussion:** This study demonstrates that acute excess energy intake may have differing effects

on an individual's DNL and IHTG, and dietary carbohydrate may impact DNL more than alcohol.

INTRODUCTION

The consumption of excess nutrients is a frequent practice of spectators at sporting events. In addition to major macronutrients (carbohydrates, fats, and proteins), alcohol is commonly consumed during such 'tailgate parties' (1, 2). World soccer and U.S. college football game tailgating can be associated not just with drinking, but with heavy or excess drinking that in some cases leads to acute adverse health consequences (2-4). For many years, two aspects of alcohol have been the focus of research - the negative social consequences of alcohol intake (3, 4), and its impact on blood lipids and cardiovascular risk (5-9). A smaller number of studies have investigated the acute effects of meals and alcohol combined on metabolism, in particular, the impacts of red wine (6, 8-10) or vodka (7, 11) with dinner. However, no studies have determined the magnitude of acute alcohol effects on intrahepatic triacylglycerols (IHTG) and the biochemical pathway of de novo lipogenesis (DNL), when combined with excess food intake. With regard to the accrual of IHTG, past research studies, conducted both in animals (Sprague Dawley rats) and humans, have tested the effects of alcohol alone (12-18), and normal meals consumed with or without alcohol (5-11, 19-21). Acutely, a bolus of alcohol in rats increased plasma triacylglycerols (TG) by 55% (12), and animals chronically treated with alcohol increased plasma TG, accumulated IHTG (16), and developed microvesicular steatosis, necrosis, and fibrosis (15, 17). In a classic study, Wiebe et al fed healthy men a bolus of alcohol (3.0 g/kg) in combination with a meal, followed by pre and post-liver biopsies, and observed significant increases in plasma liver

enzymes (aspartate aminotransferase, AST and alanine transaminase, ALT) and IHTG – both of which resolved over 24 hours (14). Siler et al (13, 18) administered an oral bolus of alcohol (vodka, 40% alcohol by volume) without food to healthy men, which led to an increase in plasma TG of 25-43% over six hours, and a 30-fold increase in DNL in TG-rich lipoproteins (TRL). Past research on hepatic DNL has focused on the stimulatory effect of positive energy balance over a period of 5 days (22) and diets high in sugars (23-26). These data have shown that the process of DNL is a key contributor to excess IHTG (27, 28), and excess consumption of food and alcohol together has the potential to stimulate DNL in a manner that would increase IHTG, especially in individuals at risk for insulin resistance who are susceptible to the fatty liver (29). Thus, although key information is available on the acute effects of alcohol on blood lipids and sugar's effects on hepatic DNL, previous study designs were not reflective of the combined intake of excess alcohol and food (5-9, 22-26). The present project was designed to investigate liver metabolism in a translational manner that is characteristic of a tailgate event. Surprisingly, we found that in overweight males, after an extended duration of eating and drinking, metabolic responses were not uniform and revealed significant individual variation in the ability to protect the liver from nutrient toxicity. These findings underscore the inter-relationships between hepatic and peripheral metabolism that can work in concert to protect individuals from the metabolic challenges brought on by over-consumption of nutrients.

MATERIALS AND METHODS

The study was approved by the University of Missouri (MU) Institutional Review Board (IRB#1211233) and registered at ClinicalTrials.gov (NCT02141880). As shown in **figure 4.1A**, 54 subjects were screened over the phone to determine preliminary eligibility. Thirty-two subjects signed the informed consent to screen in person and a total of eighteen subjects completed the in-patient protocol.

Inclusion and exclusion criteria

The inclusion criteria included sedentary men who participated in less than three hours of aerobic exercise/week, age 21-52y, BMI 25.1-51.0 kg/m², and waist girth < 55 inches, due to a limitation of the MRI scanner. Subjects reported an average of 6.6±6.2 light activity (e.g. slow walking) per week on their physical activity recall survey (30). The subjects were required to be nonsmoking with a fasting blood glucose < 125 mg/dL (non-diabetic), ALT < 40 U/L, plasma TG < 200 mg/dL, and free of metabolic disorders (thyroid and kidney conditions). Subjects were not included in the study who reported the use of prescription medication, except one person who was taking lisinopril. One subject reported the use of asthma medication as needed. To ensure the relative safety of the subjects as they ingested alcohol sufficient to maintain intoxication over a period of several hours, eligibility included greater than moderate alcohol consumption (regularly consuming alcohol in the last twelve months). A survey was used to assess typical alcohol consumption (31). Subjects were excluded if they used any tobacco products, did not regularly consume alcohol or abstained from

alcohol use, or consistently consumed more than 16 alcoholic drinks/week on average (one glass is approximately equivalent to a 12-oz beer, 4-oz glass of wine, or 1.5-oz shot of distilled spirits). Given the repeated blood draws throughout the afternoon of the study (see below), subjects were also excluded if they had donated blood within the past two months or if they had metal in their body which precluded a magnetic resonance spectroscopy (MRS) scan. Eight of the subjects completed a three-day food record (two weekdays and one weekend day) before the study (32) which revealed that they consumed $2,748 \pm 167$ kcals/day under free-living conditions (**table 4.1**).

Overall study design

As shown in **figure 4.1B**, three days prior to the study, subjects consumed, by mouth, deuterium oxide (70%, d_2O , 50 ml twice daily) to label the pathway of DNL in vivo (33). All subjects were also advised to eat their ad libitum diet for these three days. The goal was to have subjects eat a diet of typical composition and avoid novel settings of eating (buffet meals, long periods of going without food, etc.) that may alter energy balance. Subjects were advised to not consume alcohol the night before the study and avoid any acute dietary patterns that would increase lipogenesis (foods high in sugar content). Subjects were in frequent communication with research staff during these three days to assess their adherence.

Alcohol dosing protocol

The doses of alcoholic beverages were specific for each subject (formula given below) and contained 80-proof Grey Goose® vodka (40% alcohol by volume) mixed with their choice of orange juice, peach juice, or fruit punch. For each subject, the level of breath alcohol (BrAC) was collected with an Alco-Sensor® IV (Intoximeters, St. Louis, MO) starting 30 min. post-alcohol consumption and then repeated every 30 min. thereafter, with a goal of a BrAC of between 0.08-0.10 g/210L. Before each BrAC measurement, the subject rinsed their mouth with water to ensure that no alcohol residue was left in the oral cavity and a new mouthpiece was used for every measurement.

Attaining initial BrAC - For the first hour of this study, the initial dose was calculated based on the subject's height, weight, gender, and total body water (TBW) to achieve the BrAC level of 0.10 g/210L. TBW was calculated using the following formula (34): $TBW = [2.447 - 0.09156 * \text{age (y)} + 0.1074 * \text{height (cm)} + 0.3362 * \text{weight (kg)}]$. The first dose of alcohol was calculated using the following formula (35):

$$\text{Alcohol dose (g)} = [(10 * \text{BAL} * \text{TBW})/0.8] + (10 * \text{MR} * (\text{DDP} + \text{TPB})) * (\text{TBW}/0.8)] \text{ where}$$

BAL is blood alcohol level target of 0.10, MR: alcohol metabolism rate (0.015 g/100 ml TBW/hr), DDP: duration of the drinking period and in this case, the dose was calculated for the first (1) hour, and TPB: the time to peak BAL target, with the first dose goal being achieved within 1 hr. At the start of the study, the calculated first-hour dose was administered in two parts, the first half of the dose

at the start of the study (average time to complete consumption of this first drink was 16.6 min.) and the second half of the dose consumed 30 min. later.

Maintaining BrAC - After subjects had achieved the target BrAC, they were encouraged to drink in a manner to maintain this level. Considering an estimated alcohol metabolism rate of 0.015 g/100 ml TBW/hr (33), a subsequent dose of 40-50 ml was provided if BrAC was <0.10 g/210L. If the subject's BrAC level was >0.10 g/210L, no additional alcohol was added and BrAC was rechecked in 30 min.

Study day protocol

Subjects were studied in pairs, with entertainment (e.g. sports) shown on a TV to increase social interactions. As shown in **figure 4.1B**, subjects were admitted to the MU Clinical Research Center at 0800 AM. Vitals were recorded and a fasting venous blood sample was immediately obtained through an IV line. A light breakfast was served, followed by measurement of body composition by dual-energy X-ray absorptiometry (Hologic A version 13.5.2, Marlborough, MA). At ~11:00 AM, a blood sample was collected and the food and alcohol were served for the next five hours. Aside from alcohol intake, food consumption was encouraged by the replenishment of an abundant buffet made-up of highly-palatable foods (hamburgers, chips, cupcakes, etc.). The buffet food that was presented was weighed periodically (to the 0.1g) throughout the afternoon and plate waste was taken into account. For the first 10 subjects, each subject's food items were weighed separately while for the last 8 subjects, the total food

consumed by the two subjects combined was weighed/recorded and divided by two to estimate each subject's intake. The reason for this change was to reduce the likelihood of the subject feeling "under close scrutiny," which could influence his eating and drinking behavior. Nutrient intakes were calculated using the Nutrition Data System for Research (NDSR system 2019, University of Minnesota, Minneapolis, MN). During the five hours of overfeeding (from here on, referred to as treatment), blood samples were collected every hour and BrAC was measured every 30 minutes using the breathalyzer. BrAC units are reported as the weight of alcohol in grams per volume of breath (210 liters). Due to the timing and flow of measurements in the protocol, 14 of the 18 subjects underwent MRS (reproducibility: pooled standard deviation of 0.69%) (34). The scanner was a Siemens 3T Skyra series 45839 and data were analyzed by LiverLab software (Siemens Healthcare GmbH, Erlangen, Germany, R^2 fit during the study were as follows: pre - 0.92 ± 0.13 post - 0.94 ± 0.09 , mean \pm SD). The subjects remained in the clinical unit overnight where they rested and slept, each in a private room. The next day in the morning, a fasting blood sample was collected followed by measurement of energy expenditure by indirect calorimetry in the fasting state (35). The morning BrAC was confirmed to be zero before the subject was fed breakfast and discharged from the unit.

Primary and secondary outcomes

The primary outcome of this study was the measurement of IHTG before and after five hours of treatment in adult men. The secondary outcome was the

measurement of DNL in plasma TG.

Analytical methods

Labeled water (d_2O) was purchased from Cambridge Isotope Laboratory, Inc. (Purity $\geq 99.5\%$, Catalog# DLM-4-70-0, Andover, MA) and final d_2O enrichments in plasma were measured by cavity ringdown spectroscopy using a Liquid Water Isotope Analyzer with automated injection system, version 2 upgrade (Los Gatos Research, Mountain View, CA) by Metabolic Solutions Inc. Nashua (NH). This timing of d_2O consumption resulted in a body d_2O enrichment of $0.32 \pm 0.07\%$ (mean \pm SD), which is similar to that achieved in past studies (33). To maintain body d_2O enrichments during the study, deuterium oxide was also added to the alcoholic drinks. After treatment was concluded, plasma samples were immediately processed to isolate TRL using ultracentrifugation at 40,000 rpm at $15^\circ C$ in a 50.3Ti rotor (Beckman Instruments, Palo Alto, CA) for 20h (36). The TRL-TG were separated via thin-layer chromatography and fatty acid methyl esters prepared as described previously (36). Labeled fatty acids were measured using a 6890N gas chromatography coupled to a 5975 mass spectrophotometry detector (Agilent Technologies, Palo Alto, CA) and DNL calculated by mass isotopomer distribution analysis (37). In the postprandial state, TRL particles include both chylomicrons and VLDL. Assuming intestinal DNL is minimal (23) then the apparent fractional DNL in TRL may be lower than DNL analyzed only in VLDL. Absolute DNL is calculated by multiplying the

fractional DNL in TG by the quantity of TG (38) and thus, absolute DNL reflects liver fatty acid synthesis and secretion (in mg/dL).

Plasma concentrations of TC, TG, LDLc, HDLc, AST, and ALT were measured by a CLIA-standardized laboratory (Quest Diagnostics, St. Louis, MO, Lic. #26D0652092). The measurements of lipids were performed via auto-analyzer (Roche Cobas 8000 System, CV 0.6-0.9%, Indianapolis, IN) using electro-chemiluminescent immunoassay. Liver enzymes were measured using UV Absorbance (Roche Cobas 8000 System, CV 0.5-3.2% for AST and 0.5-3.1% for ALT, Indianapolis, IN). Assay kits were used to measure the concentrations of plasma glucose (Wako #439-90901, CV 6.6%, Mountain View, CA) and NEFA (Wako #991-34891, CV 6.9%, Mountain View, CA). Plasma insulin was measured using an enzyme-linked immunosorbent assay (Human Insulin, EMD Millipore #EZHI-14K, CV 7.2%, Billerica, MA).

Statistical analysis and calculations

Subject characteristics are presented as mean \pm SD, while the effects of the five-hour treatment over time are presented as mean \pm SEM. Basal metabolic rate was calculated using the Harris Benedict equation (39) and total energy needs were calculated by multiplying basal metabolic rate by the individual subject's physical activity factor (average physical activity factor was 1.3 \pm 0.1). A paired sample t-test was performed using StatView®, 5.0.1 software (v2008), regression analyses using the Statistical Package for the Social Sciences (SPSS®, v24,

2016), and Pearson correlation analysis using SPSS® (v24, 2016). HOMA-IR was calculated as $[(\text{glucose in mg/dL} \times \text{insulin in } \mu\text{U/mL})/405]$. The nonalcoholic fatty liver disease (NAFLD) fibrosis score was calculated using the formula (40):

$$\text{NAFLD Fibrosis score} = -1.675 + [0.037 \times \text{age (years)}] + [0.094 \times \text{BMI (kg/m}^2)] + [1.13 \times \text{hyperglycemia or diabetes (yes = 1, no = 0)}] + [0.99 \times \text{AST/ALT ratio}] - [0.013 \times \text{platelet (} \times 10^9 \text{/L)}] - [0.66 \times \text{albumin (g/dL)}],$$

and FIB-4 was calculated using the formula (41):

$$\text{FIB-4} = [\text{age (years)} \times \text{AST (U/L)}] / [\text{platelet (} 10^9 \text{/L)} \times \sqrt{\text{ALT (U/L)}}].$$

RESULTS

Table 4.2 presents the subject characteristics and shows that the subjects ranged from overweight to obese, and had healthy levels of glucose, lipids, and liver enzymes. According to the entry criteria, each subject was a habitual consumer of alcohol and had consumed more than six drinks in at least one setting within the past 30 days. The levels of IHTG ranged from very low to elevated (1.6 – 22.9%). During the five hours of food and alcohol treatment, subjects consumed $5,087 \pm 632$ kcal or $191 \pm 25\%$ of their calculated (39) total daily energy needs (i.e., $2,690 \pm 369$). The composition of the energy taken in during treatment was 32% from carbohydrates, 35% from fat, 10% from protein, and 23% from alcohol. The total alcohol intake was 171 ± 24 g, which resulted in an average BrAC level of 0.08 ± 0.00 (range 0.05-0.09) g/210L (**fig. 4.2A**) and subjective intoxication level of 3.9 ± 0.4 (range 1.6-4.6, **fig. 4.2B**).

Biochemical measurements and de novo lipogenesis, and liver fat

As shown in **figure 4.2C-F**, treatment significantly increased plasma insulin from 10 ± 3 U/L at fasting to 71 ± 17 U/L after eating ($P=0.003$), plasma glucose from 91 ± 3 mg/dL to 113 ± 5 mg/dL ($P<0.001$), plasma TG from 98 ± 8 mg/dL to 358 ± 40 mg/dL ($P<0.001$), and reduced NEFA concentrations from 0.47 ± 0.05 mmol/L to 0.40 ± 0.06 mmol/L ($P<0.001$). The next morning, values returned to a normal for plasma concentrations of glucose (94 ± 17 mg/dL, $P=0.300$, **fig. 4.2D**), NEFA (0.47 ± 0.05 mmol/L, $P=0.828$, **figure 4.2E**), and TG (135 ± 20 mg/dL, $P=0.053$, **fig. 4.2F**). Fasting plasma insulin remained slightly higher (16 ± 4 U/L, $P=0.011$, **fig.**

4.2C) than it had been the previous morning. Shown in **figure 4.3A-C** are the significant increases observed for TRL-TG (60 ± 8 mg/dL to 208 ± 31 mg/dL, $P<0.001$), and both percent DNL ($11\pm 2\%$ to $17\pm 2\%$, $P<0.001$) and absolute DNL (7 ± 2 mg/dL to 34 ± 6 mg/dL, $P<0.001$). As shown in **figure 4.3D**, for the group as a whole, no significant changes were observed in total IHTG after treatment ($9.1\pm 1.8\%$ to $9.3\pm 1.9\%$, $P=0.670$). However, when the absolute change in IHTG is plotted individually for the 15 subjects who underwent MRS (**fig. 4.3E**) varying responses were observed: Nine subjects increased IHTG, five subjects decreased, and one did not change IHTG. Correlation analysis (**fig. 4.4A**) revealed a significant positive relationship between the absolute increase in IHTG and an increase in the fraction of TRL-TG originating from DNL ($r=0.617$, $P=0.014$).

Group differences

When subjects were divided into two groups based on their change in IHTG, DNL was the only predictor of the differences between the groups when considered as either fractional lipogenesis (**fig. 4.4A**, $P=0.006$ for the entire effect of time, or **fig. 4.4B**, $P=0.018$, when comparing just the 5h time point) or as absolute DNL (**fig. 4.4C**). The change in TRL-TG was not different between the groups (**fig. 4.4D**) and no significant differences were observed for the total amount of energy consumed by each group (**fig. 4.4E**), although the group with reduced IHTG tended to consume less energy from non-alcohol sources (i.e. 15.7% fewer calories, $P=0.076$, from carbohydrate, fats, and proteins) and needed significantly

more alcohol to achieve BrAC of 0.08 g/210L. Note that the study design aimed to have all subjects attain a BrAC of less than 0.10 g/210L and this was achieved equally in both groups (**fig. 4.4F**). However, the amount of alcohol needed to reach steady-state BrAC was 2.50 g/kg alcohol in those whose IHTG decreased and 1.32 g/kg alcohol in those whose IHTG increased ($P=0.048$, data not shown). As shown in **figure 4.5**, other variables that were tested and failed to predict the change in IHTG included plasma concentrations of insulin, NEFA, and TG. Glucose tended to be higher during treatment in the group that reduced IHTG (**fig. 4.5D** and the higher the glucose the more IHTG was reduced, $r=0.747$, $P=0.074$, data not shown). No relationship was found for baseline IHTG, even when correlation analysis was performed for all subjects (data not shown). The groups were not different with respect to free-living, ad libitum alcohol intake (data not shown). Lastly, as shown in **figure 4.5E**, although the whole-body fat oxidation rates measured the next morning were 354% higher in the group with reduced IHTG, no significant differences were observed between the groups due to high variability (**fig. 4.5F**, $P=0.259$).

DISCUSSION

The goal of the present study was to determine the metabolic impact of excess energy consumption that included both food and alcohol intake, in a situation that mimicked celebrations associated with sporting events. The primary outcome of the study was the measurement of IHTG before and after this treatment in adult men. Contrary to our hypothesis, five hours of eating and drinking alcohol did not lead to liver fat accumulation when data from all subjects were combined. These results are not consistent with previous literature conducted both in animals and humans (14, 16), most likely because the present subjects consumed a lower amount of ethanol, 1.7 ± 0.2 g/kg over a five-hour period, compared to past research. In Sprague-Dawley rats, Brodie et al (16) reported a significant increase in IHTG peaking 15-20 hours after a 4.8g/kg bolus of alcohol was given. Similar outcomes were observed in the study conducted by Wiebe et al using repeat liver biopsies over a 24h period in adult men after they had consumed ethanol (3 g/kg, which we estimate equaled a total of 210g) and a 300 kcal meal (14). The protocol of this present study maintained BrAC less than 0.10 g/210L (final values were 0.08 ± 0.00 g/210L), while in the study of Wiebe et al (14), subject blood alcohol concentration was 1.3-1.8 parts per thousand (equivalent to BrAC of 0.13-0.18 g/210L) within six hours. Additionally, unlike the previous studies (14, 16), the subjects' energy consumption levels were very high, including $3,890 \pm 142$ kcals from non-alcoholic sources. In a study by Rubin et al (20), subjects consumed both food and alcohol, and the multi-day protocol incorporated energy from alcohol being either substituted isocalorically (68-130

g/d) for carbohydrates or added to their standard diet (180-270 g/d). In either case, subjects increased IHTG by two to four times and this occurred as early as two days of alcohol administration. Thus, although unlikely, the lack of change in IHTG in a subset of the present subjects who consumed 171 ± 24 g of alcohol may have been due to an insufficient time for these subjects to accumulate IHTG.

The secondary goal of this study was to measure changes in DNL in response to five hours of treatment. Increased DNL is a key distinguishing characteristic in patients with NAFLD (28), however, no studies in the past have investigated the effects on DNL of food and alcohol consumed together. In a previous set of studies (13, 18), Siler et al tested the isolated effects of 24g and 48g of alcohol (no food) administered in four doses over two hours (13) or two doses over half an hour (18). The increases in VLDL-TG DNL, peaking at $37\pm 7\%$ (13) and $30\pm 8\%$ (18), were higher than the increase observed here ($20\pm 8\%$) after we administered a greater quantity of alcohol (171 ± 24 g) over five hours. Lower observed fractional DNL in the present study results from DNL being measured in the TRL fraction, which contains both VLDL and chylomicrons, instead of VLDL alone (13, 18). Because chylomicron-TG primarily carry dietary sources of fatty acids, DNL fatty acids measured in TRL are diluted by unlabeled chylomicron-TG. Interestingly, in the present group as a whole, only the amount of alcohol consumed during five hours was found to be significantly related to the increase in percent DNL ($r=0.734$, $P=0.038$, data not shown).

With regard to biochemical changes, as expected, significant increases were observed for plasma glucose and TG while NEFA concentrations decreased significantly. These changes are consistent with previous studies in which alcohol was fed alone (13) or with meals (5, 7-11). A study conducted by Fielding et al (11) in seven men consuming a bolus of vodka (47.5 g) with a meal high in carbohydrate reported a significant increase in large VLDL-TG particles (Svedberg flotation rate, S_f 60-400), but not small VLDL (S_f 20-60) and chylomicrons ($S_f > 400$). These data suggest that newly-made fatty acids add to the size of the VLDL particle such that the significant increase in DNL observed here may have led to the secretion of larger VLDL particles and therefore higher TRL-TG concentrations.

Regarding changes in IHTG, Rubin et al (20) reported that three out of four subjects, in a group who received alcohol in addition to their study diet, failed to exhibit any change in the histological measures of steatosis which suggests individual variability in response to a meal and alcohol. We also found a wide variation in the response of subjects after consuming food and alcohol. Of the 15 subjects who underwent liver scanning, nine increased IHTG, five subjects decreased IHTG, and one subject did not change. When subjects were categorized by IHTG response (increased IHTG, $n=9$; and decreased IHTG, $n=5$), changes in DNL were also significantly different between the groups. Consistent with our previous findings (28), individuals with increased IHTG exhibited higher DNL, while those whose IHTG was reduced exhibited lower DNL

values when expressed as AUC₀₋₅ or postprandial average over five hours. Subjects with increased IHTG also had lower plasma glucose concentrations suggesting glucose was readily utilized in the DNL pathway, although insulin levels were not different between groups, (**fig. 4.5A**). Interestingly, the group with increased IHTG consumed significantly less alcohol compared to the other group (1.32 g/kg vs 2.50 g/kg, $P=0.048$) and tended to consume 6% more energy from carbohydrates ($P=0.128$). The BrAC was not different between the groups during treatment (by design), which means that subjects with increased IHTG needed less alcohol to achieve a BrAC of 0.08 g/210L. We did not find a difference in ad libitum alcohol intake between the groups, but it is possible that the subjects who needed less alcohol to maintain BrAC, were less able to oxidize, resulting in a build-up in alcohol in the blood and in the liver, routing of this substrate to storage. Thus, the protective nature of the group of individuals whose IHTG decreased could be partially attributed to higher whole-body fatty acid oxidation rates. Significance was not achieved between groups due to smaller sample sizes and this needs to be confirmed in future studies. Lastly, a potential explanation of these findings is that high carbohydrate consumption may have a greater impact on liver fat than alcohol in some subjects. Further studies are needed in a larger population to understand these differences between individuals in response to excess food and alcohol and their impact on DNL and IHTG.

The primary limitation of this study was the inclusion of only men as subjects which was due to a limit on the total number of subjects that could be studied using an intensive protocol and limited funding. In an early report by Taylor et al, alcohol consumption was associated with increased serum-TG levels in males but not in females (42) and the present data will be used to design future studies with a larger sample size to accommodate individual variability in response and sex as a biological variable. Further, the focus of the current study was to understand the liver's response to excess food and alcohol intake by conducting a protocol that mimicked real-life events, while maintaining scientific rigor. Although food intake was in vast excess above the subjects' daily needs, no data exist in the literature on the average food and beverage intake of spectators before and after sporting events and there are undoubtedly some individuals who eat and drink in a manner similar to this study. Given the limited funding, it was not feasible for us to collect subjects' genotype information and measure glucose tolerance during screening. Due to safety concerns, the goal was for subjects to achieve a BrAC below 0.10 g/210L, while under real-world tailgate conditions, some individuals drink more and some less. As a safety measure, plasma ALT was quantitated to assess liver health at the end of the 24-hour study and the values were similar to the previous day's fasting concentration. These data, and that from the literature (14, 20), suggest that the livers of relatively young, healthy individuals may accommodate large excesses in nutrient intake, given time to recover.

CONCLUSIONS

Our previous cross-sectional observations of significant positive relationships between hepatic fatty acid synthesis and high liver fat (27, 28) are echoed in the results of the present study, even though these data were generated after a single bout of overeating. Overconsumption of food and alcohol significantly increased IHTG in some subjects and these individuals required less alcohol consumption to achieve the same BrAC, they tended to consume more dietary carbohydrates, and increased liver DNL to a greater extent.

FUTURE DIRECTIONS

The primary goal of this study was to determine the acute effect of combined food and alcohol intake (similar to tailgating) on liver fat measurements. Although, both alcohol and carbohydrates have shown to stimulate DNL, the results from this study hinted toward a major role of carbohydrates rather than alcohol when consumed in combination. Given the high prevalence of overconsumption of food and alcohol in the U.S., further studies are warranted to better understand the interactions between personal consumption habits and individual metabolic variation in handling excess nutrients.

Table 4.1: Dietary intake in a subset of subjects' ad libitum days for comparison to the study day

Macronutrient	Ad libitum	Study day	P-value
Total energy (kcal)	2748 ± 472	4875 ± 786	0.001
Carbohydrates			
Grams	320 ± 62	336 ± 58	0.688
Kcals	1281 ± 249	1342 ± 231	0.688
Percent	47 ± 7	28 ± 6	0.002
Proteins			
Grams	106 ± 23	116 ± 19	0.303
Kcals	390 ± 143	462 ± 75	0.208
Percent	14 ± 4	10 ± 3	0.042
Fat			
Grams	111 ± 24	180 ± 29	< 0.001
Kcals	1002 ± 212	1618 ± 263	< 0.001
Percent	36 ± 4	34 ± 7	0.133
Alcohol			
Grams	11 ± 18	207 ± 149	0.008
Kcals	75 ± 128	1452 ± 1041	0.008
Percent	3 ± 4	28 ± 15	0.003

Legend: Data from three-day food records were collected and analyzed by NDSR®. Data are presented in mean±SD. P-value for paired t-test, n=8.

Table 4.2: Baseline subjects characteristics

Age and alcohol intake	n=18	Range
Age (y)	31.4 ± 7.3	[21 – 47]
Alcohol intake		
Maximum drinks in one occasion (30 days)	6.5 ± 5.3	[1.0 – 18.0]
Maximum drinks in one occasion (12 months)	10.1 ± 6.0	[2.0 – 20.0]
Average drinking days/week (3 months)	1.9 ± 1.3	[0.3 – 3.5]
Anthropometrics and blood pressure		
Height (m)	1.77 ± 0.08	[1.60 – 1.87]
Weight (kg)	100.7 ± 15.2	[75.1 – 129.0]
BMI (kg/m ²)	32.1 ± 5.9	[26.3 – 50.4]
Waist (cm) ^a	109.5 ± 13.1	[93.0 – 138.0]
Body composition		
Body fat (kg)	32.7 ± 8.8	[21.8 – 55.8]
Body fat (%)	34.2 ± 8.6	[24.0 – 62.5]
Trunk fat mass (kg)	22.7 ± 14.8	[9.5 – 64.1]
Trunk fat mass (%)	22.3 ± 13.3	[10.7 – 51.6]
Visceral adipose tissue (g) ^c	585 ± 209	[324 – 1058]
Lean mass (kg)	62.2 ± 12.3	[24.3 – 81.2]
Lean mass (%)	64.4 ± 4.9	[53.5 – 72.5]
Trunk lean mass (kg)	40.0 ± 6.3	[13.3 – 44.8]
Trunk lean mass (%)	39.6 ± 4.7	[17.7 – 37.4]
Blood pressure		
Systolic blood pressure (mmHg) ^d	127 ± 10	[104 – 148]
Diastolic blood pressure (mmHg) ^d	81 ± 10	[65 – 105]
Heart rate (bpm) ^d	70 ± 12	[58 – 100]
Biochemical measurements		
Plasma glucose (mg/dL) ^d	91 ± 10	[73 – 111]
Plasma insulin (U/L) ^d	10 ± 12	[1 – 51]
NEFA (mmol/L) ^d	0.47 ± 0.22	[0.18 – 0.85]
HOMA-IR ^d	2.1 ± 2.5	[0.2 – 10.7]
Lipids		
Total cholesterol (mg/dL)	173 ± 28	[135 – 255]
Plasma triacylglycerols (mg/dL) ^d	98 ± 32	[47 – 147]
HDL cholesterol (mg/dL)	46 ± 10	[28 – 66]
LDL cholesterol (mg/dL)	107 ± 25	[57 – 171]
Liver-related measurements		
IHTG (%) ^b	9.1 ± 6.9	[1.6 – 22.9]
AST (U/L)	24.9 ± 5.7	[17.0 – 40.0]
ALT (U/L)	29.5 ± 10.0	[15.0 – 50.0]
ALP (U/L)	69.5 ± 20.8	[32.0 – 127.0]
Albumin (g/dL)	4.7 ± 0.3	[4.1 – 5.0]
NAFLD Fibrosis Score	-2.6 ± 1.6	[-6.0 – -0.1]
FIB-4	0.65 ± 0.34	[0.22 – 1.63]

Legend: Data are reported in mean±SD, n=18 males unless otherwise noted (race categories: 14 white, 3 black and 1 other). a n=14, b n=15, c n=16, d n=17.

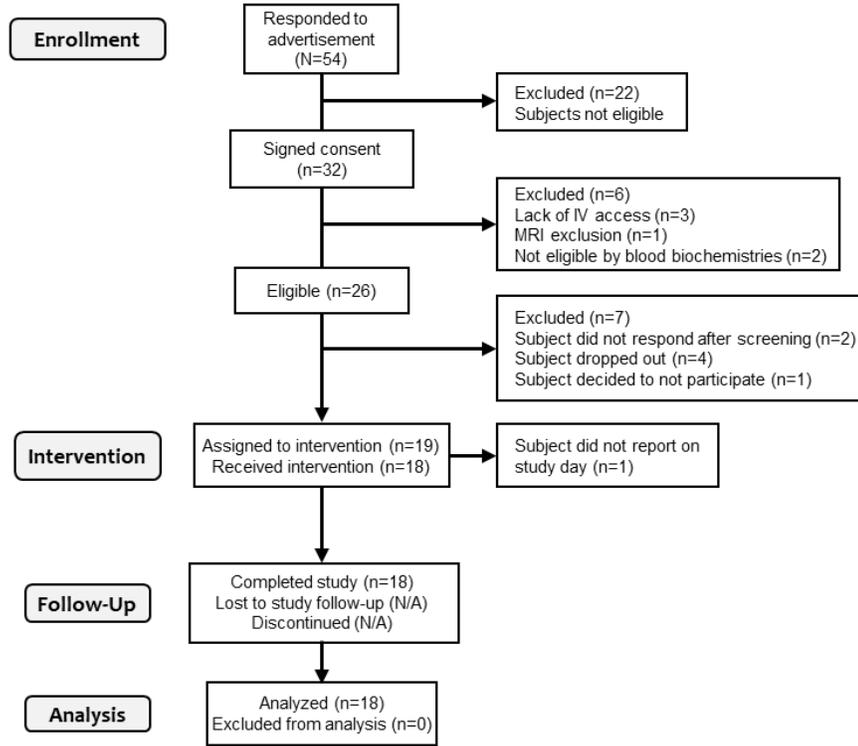
Abbreviations: BMI, body mass index; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; NEFA, nonesterified fatty acids; HOMA-IR, homeostatic model assessment for insulin resistance; IHTG, intrahepatic triacylglycerols; AST, aspartate aminotransferase/serum glutamic-oxaloacetic transaminase; ALT, alanine aminotransferase/serum glutamic pyruvic transaminase; ALP, alkaline phosphatase. HOMA-IR was calculated using the formula: [plasma glucose (mg/dL) * Insulin (U/L)]/405.

Figure 4.1: Study consort flow diagram and protocol timeline

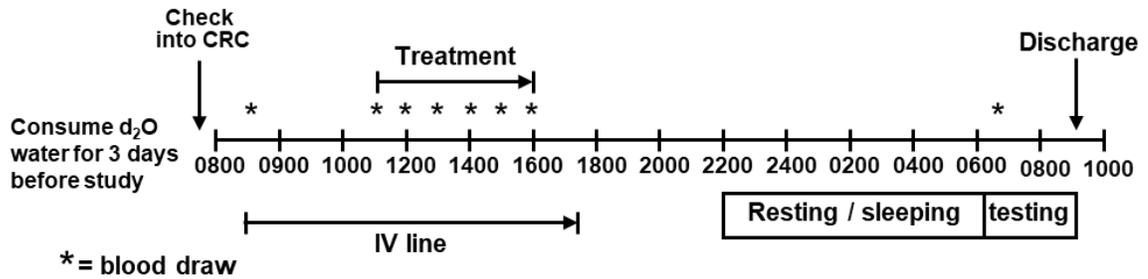
4.1A

CONSORT

TRANSPARENT REPORTING of TRIALS



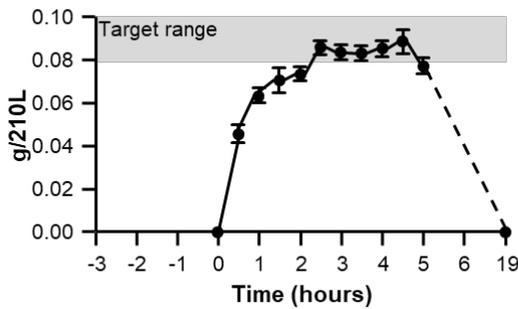
4.1B. Study timeline



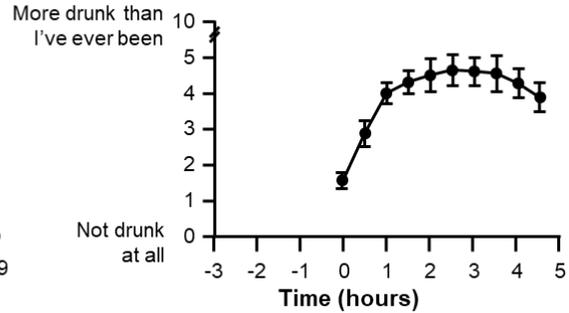
Legend: Abbreviations: CRC, clinical research center; d₂O, deuterated water.

Figure 4.2: Breath alcohol concentrations, subjective intoxication, plasma insulin and metabolites

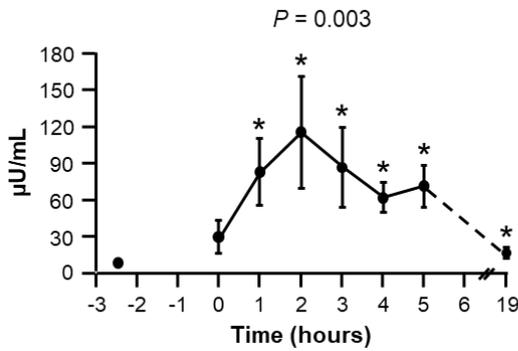
4.2A. Breath alcohol content



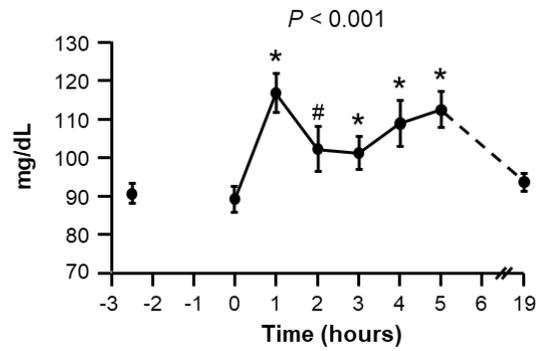
4.2B. Subjective intoxication



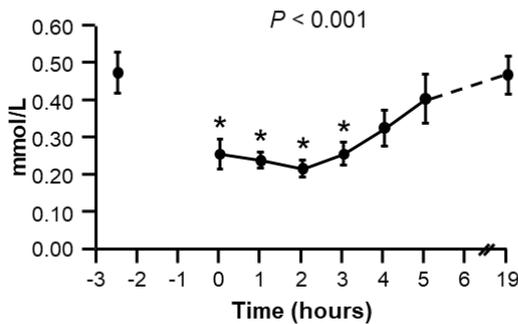
4.2C. Insulin



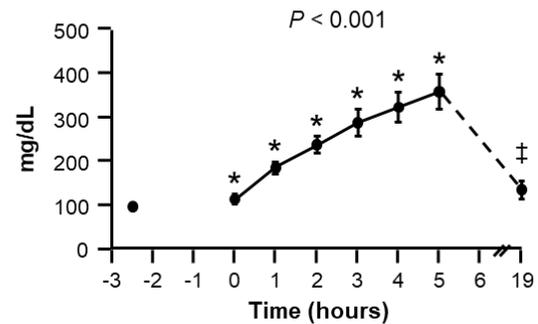
4.2D. Glucose



4.2E. NEFA



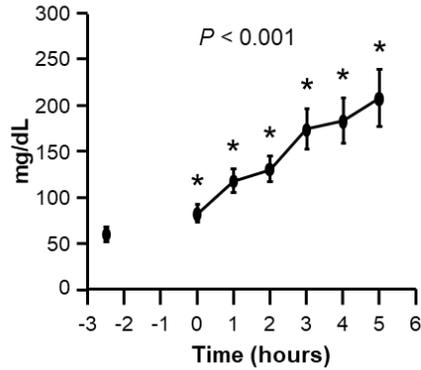
4.2F. Triacylglycerols



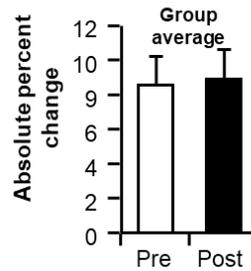
Legend: Data are reported in mean \pm SEM. *P*-value for ANOVA effect of time, and * $P \leq 0.05$, # $P = 0.08$, † $P = 0.053$ compared to fasting. In the figures, time zero represents the value immediately before treatment began; please see methods for the alcohol dosing regimen. The 19-hour time point represents a measurement performed in the fasted state the next morning.

Figure 4.3: Changes in DNL and liver fat

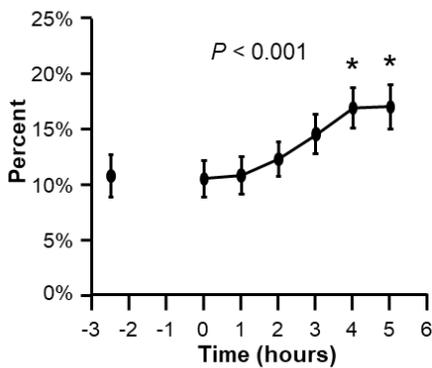
4.3A. TRL-TG



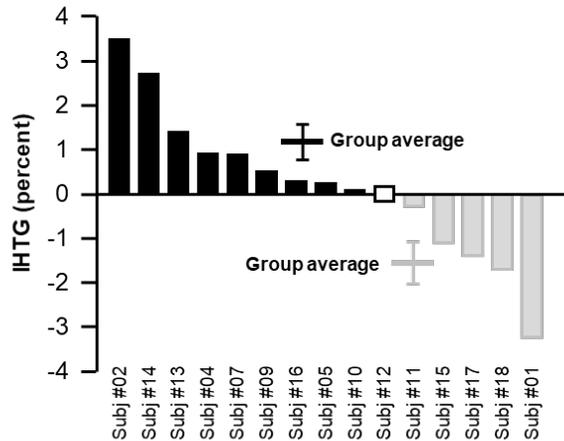
4.3D. Change in IHTG for all subjects



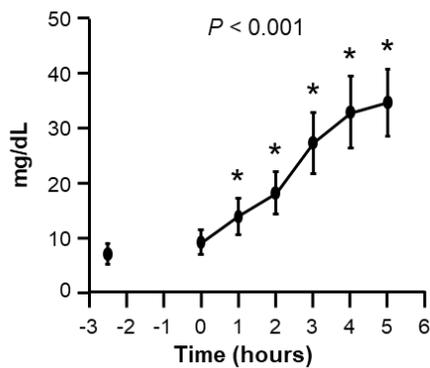
4.3B. Percent DNL



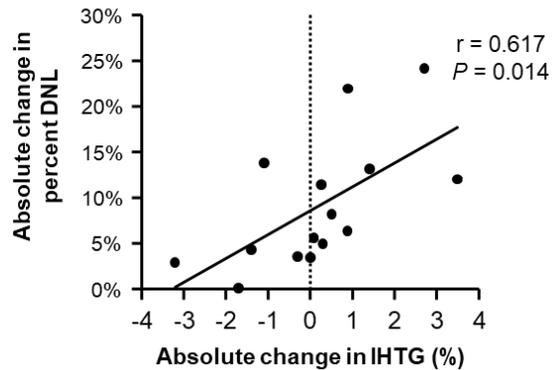
4.3E. Absolute change in individual IHTG



4.3C. Absolute DNL



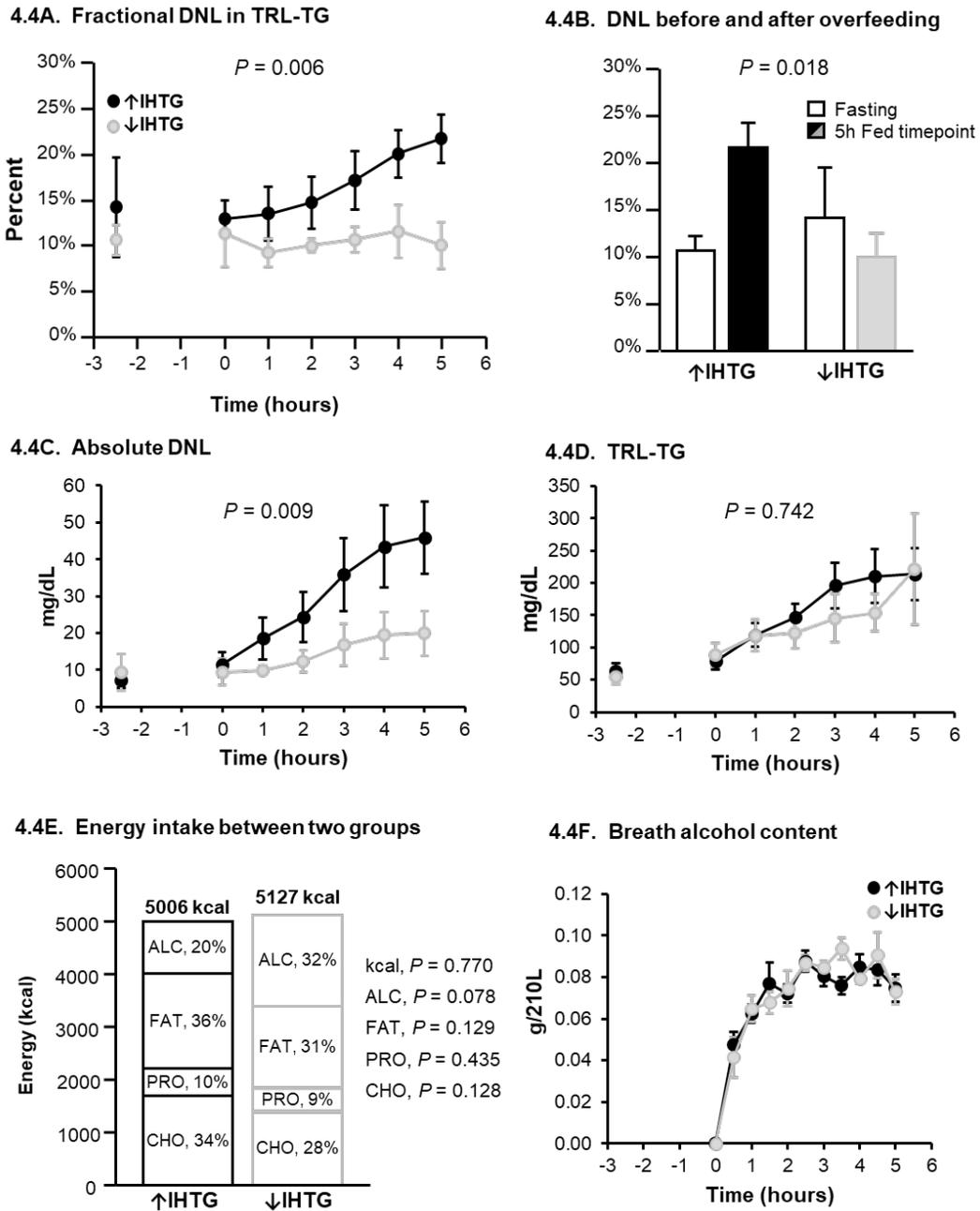
4.3F. Relationship between DNL and IHTG



Legend: Data are mean±SEM, *P*-value for ANOVA effect of time. * *P*≤0.05 compared to fasting. Time zero represents the value immediately before treatment began. In **4.3D**, Pre and Post represent the timing of the liver MRS immediately before and after treatment. **4.3E** shows individual data on change in IHTG due to treatment. Of the 15 subjects who underwent repeat MRS, nine increased IHTG (black-filled bars), one showed no change (unfilled bar), and five subjects decreased IHTG (grey-filled bars). The horizontal lines represent the

group mean \pm SEM ($P=0.001$). Abbreviations: TRL, triacylglycerol-rich lipoprotein; DNL, de novo lipogenesis, IHTG, intrahepatic TG.

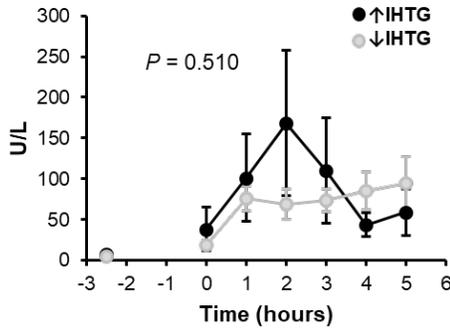
Figure 4.4: Group differences in DNL, TRL-TG, food intake, and breath alcohol concentrations



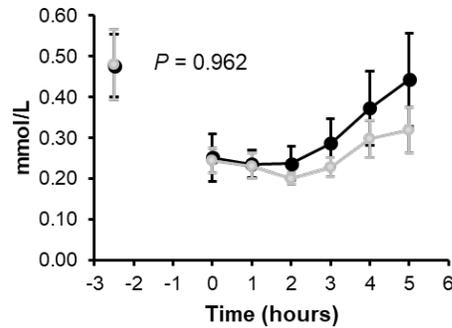
Legend: Data are reported in mean±SEM for the nine individuals who increased IHTG after five hours after treatment (black symbols) and the five individuals who decreased IHTG five hours after treatment (grey-symbols). **4.4E** shows food and alcohol intake during the 5h treatment as analyzed by NDSR®. **4.4F**, no differences were found in the rates at which the groups achieved a steady state of breath alcohol content. Abbreviations: DNL, de novo lipogenesis; IHTG, intrahepatic triacylglycerols.

Figure 4.5: Plasma insulin and blood metabolites, and fasting whole-body, fat oxidation

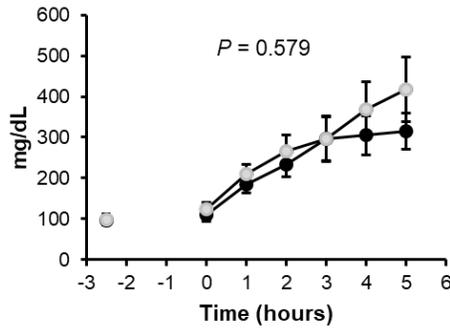
4.5A. Insulin



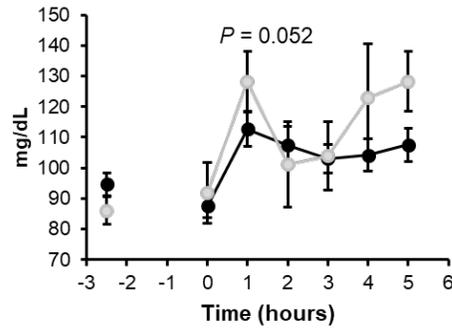
4.5B. NEFA



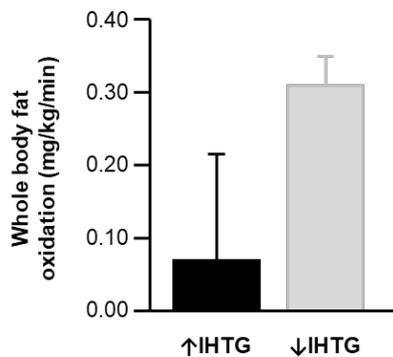
4.5C. Triacylglycerols



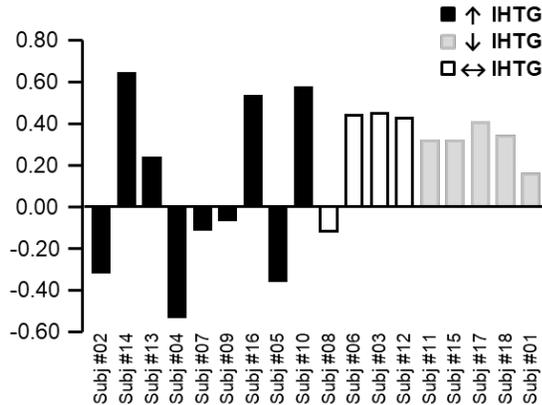
4.5D. Glucose



4.5E. Group averages in fat oxidation



4.5F. Individual fat oxidation rates



Legend: Data are mean±SEM, n=18. Abbreviations: DNL, de novo lipogenesis; IHTG, intrahepatic triacylglycerols. Black symbols represent data from nine individuals who increased IHTG after five hours after treatment; grey symbols represent data from five individuals who decreased IHTG five hours after treatment. Fasting, whole-body fat oxidation was assessed the morning after treatment. **4.5E**, the t-test performed between the two groups showed no significant difference (P=0.259). **4.5F** shows that in 6 subjects, fat oxidation rates were calculated to be negative, which indicates that DNL was ongoing.

REFERENCES

1. Neighbors, C., Oster-Aaland, L., Bergstrom, R. L., & Lewis, M. A. (2006). Event- and context-specific normative misperceptions and high-risk drinking: 21st birthday celebrations and football tailgating. *J Stud Alcohol*, 67(2), 282-289. doi:10.15288/jsa.2006.67.282. **PMC2459312**.
2. Neal, D. J., & Fromme, K. (2007). Hook 'em horns and heavy drinking: alcohol use and collegiate sports. *Addict Behav*, 32(11), 2681-2693. doi:10.1016/j.addbeh.2007.06.020. **PMC2527738**.
3. Leavens, E. L. S., Croff, J., Feddor, R., & Olson, K. (2019). It's game time: drinking intentions, alcohol consumption, and consequences at college tailgates. *Subst Use Misuse*, 54(1), 11-17. doi:10.1080/10826084.2018.1485698.
4. Glassman, T., Werch, C. E., Jobli, E., & Bian, H. (2007). Alcohol-related fan behavior on college football game day. *J Am Coll Health*, 56(3), 255-260. doi:10.3200/JACH.56.3.255-260.
5. Chung, B. H., Doran, S., Liang, P., Osterlund, L., Cho, B. H., Oster, R. A., Darnell, B., & Franklin, F. (2003). Alcohol-mediated enhancement of postprandial lipemia: a contributing factor to an increase in plasma HDL and a decrease in risk of cardiovascular disease. *Am J Clin Nutr*, 78(3), 391-399. doi:10.1093/ajcn/78.3.391.
6. Fuhrman, B., Lavy, A., & Aviram, M. (1995). Consumption of red wine with meals reduces the susceptibility of human plasma and low-density lipoprotein to lipid peroxidation. *Am J Clin Nutr*, 61(3), 549-554. doi:10.1093/ajcn/61.3.549.
7. Hampton, S. M., Isherwood, C., Kirkpatrick, V. J., Lynne-Smith, A. C., & Griffin, B. A. (2010). The influence of alcohol consumed with a meal on endothelial function in healthy individuals. *J Hum Nutr Diet*, 23(2), 120-125. doi:10.1111/j.1365-277X.2009.01021.x.
8. van Tol, A., van der Gaag, M. S., Scheek, L. M., van Gent, T., & Hendriks, H. F. (1998). Changes in postprandial lipoproteins of low and high density caused by moderate alcohol consumption with dinner. *Atherosclerosis*, 141 Suppl 1, S101-103. doi:10.1016/s0021-9150(98)00226-3.
9. Hendriks, H. F., Veenstra, J., van Tol, A., Groener, J. E., & Schaafsma, G. (1998). Moderate doses of alcoholic beverages with dinner and postprandial high density lipoprotein composition. *Alcohol Alcohol*, 33(4), 403-410. doi:10.1093/oxfordjournals.alcalc.a008410.
10. van der Gaag, M. S., Sierksma, A., Schaafsma, G., van Tol, A., Geelhoed-Mieras, T., Bakker, M., & Hendriks, H. F. (2000). Moderate alcohol consumption and changes in postprandial lipoproteins of premenopausal and postmenopausal women: a diet-controlled, randomized intervention study. *J Womens Health Gen Based Med*, 9(6), 607-616. doi:10.1089/15246090050118134.
11. Fielding, B. A., Reid, G., Grady, M., Humphreys, S. M., Evans, K., & Frayn, K. N. (2000). Ethanol with a mixed meal increases postprandial triacylglycerol but decreases postprandial non-esterified fatty acid

- concentrations. *Br J Nutr*, 83(6), 597-604.
doi:10.1017/s0007114500000763.
12. Chang, G. Q., Karatayev, O., Ahsan, R., Avena, N. M., Lee, C., Lewis, M. J., Hoebel, B. G., & Leibowitz, S. F. (2007). Effect of ethanol on hypothalamic opioid peptides, enkephalin, and dynorphin: relationship with circulating triglycerides. *Alcohol Clin Exp Res*, 31(2), 249-259.
doi:10.1111/j.1530-0277.2006.00312.x.
 13. Siler, S. Q., Neese, R. A., Parks, E. J., & Hellerstein, M. K. (1998). VLDL-triglyceride production after alcohol ingestion, studied using [2-¹³C] glycerol. *J Lipid Res*, 39(12), 2319-2328.
 14. Wiebe, T., Lundquist, A., & Belfrage, P. (1971). Time-course of liver fat accumulation in man after a single load of ethanol. *Scand J Clin Lab Invest*, 27(1), 33-36. doi:10.3109/00365517109080186.
 15. Oliva, J., French, B. A., Li, J., Bardag-Gorce, F., Fu, P., & French, S. W. (2008). Sirt1 is involved in energy metabolism: the role of chronic ethanol feeding and resveratrol. *Exp Mol Pathol*, 85(3), 155-159.
doi:10.1016/j.yexmp.2008.08.002. **PMC2874466**.
 16. Brodie, B. B., Butler, W. M., JR., Horning, M. G., Maickel, R. P., & Maling, H. M. (1961). Alcohol-induced triglyceride deposition in liver through derangement of fat transport. *The American Journal of Clinical Nutrition*, 9(4), 432-435. doi:10.1093/ajcn/9.4.432.
 17. Barson, J. R., Karatayev, O., Chang, G. Q., Johnson, D. F., Bocarsly, M. E., Hoebel, B. G., & Leibowitz, S. F. (2009). Positive relationship between dietary fat, ethanol intake, triglycerides, and hypothalamic peptides: counteraction by lipid-lowering drugs. *Alcohol*, 43(6), 433-441.
doi:10.1016/j.alcohol.2009.07.003. **PMC2758659**.
 18. Siler, S. Q., Neese, R. A., & Hellerstein, M. K. (1999). De novo lipogenesis, lipid kinetics, and whole-body lipid balances in humans after acute alcohol consumption. *Am J Clin Nutr*, 70(5), 928-936. doi:10.1093/ajcn/70.5.928.
 19. Gin, H., Morlat, P., Ragnaud, J. M., & Aubertin, J. (1992). Short-term effect of red wine (consumed during meals) on insulin requirement and glucose tolerance in diabetic patients. *Diabetes Care*, 15(4), 546-548.
doi:10.2337/diacare.15.4.546.
 20. Rubin, E., & Lieber, C. S. (1968). Alcohol-induced hepatic injury in nonalcoholic volunteers. *N Engl J Med*, 278(16), 869-876.
doi:10.1056/NEJM196804182781602.
 21. Clevidence, B. A., Reichman, M. E., Judd, J. T., Muesing, R. A., Schatzkin, A., Schaefer, E. J., Li, Z., Jenner, J., Brown, C. C., Sunkin, M., & et al. (1995). Effects of alcohol consumption on lipoproteins of premenopausal women. A controlled diet study. *Arterioscler Thromb Vasc Biol*, 15(2), 179-184. doi:10.1161/01.atv.15.2.179.
 22. Schwarz, J.-M., Neese, R. A., Turner, S., Dare, D., & Hellerstein, M. K. (1995). Short-term alterations in carbohydrate energy intake in humans: striking effects on hepatic glucose production, de novo lipogenesis, lipolysis, and whole-body fuel selection. *J Clin Invest*, 96, 2735-2743.

23. 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. doi:10.1093/ajcn/81.1.35.
24. Parks, E. J. (2001). Effect of dietary carbohydrate on triglyceride metabolism in humans. *J Nutr*, 131(10), 2772S-2774S. doi:10.1093/jn/131.10.2772S.
25. Hudgins, L. C., Hellerstein, M. K., Seidman, C. E., Neese, R. A., Tremaroli, J. D., & Hirsch, J. (2000). Relationship between carbohydrate-induced hypertriglyceridemia and fatty acid synthesis in lean and obese subjects. *J Lipid Res*, 41(4), 595-604.
26. Chong, M. F., Fielding, B. A., & Frayn, K. N. (2007). Mechanisms for the acute effect of fructose on postprandial lipemia. *Am J Clin Nutr*, 85(6), 1511-1520.
27. Donnelly, K. L., Smith, C. I., Schwarzenberg, S. J., Jessurun, J., Boldt, M. D., & Parks, E. J. (2005). Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J Clin Invest*, 115(5), 1343-1351. doi:10.1172/JCI23621. **PMCID: PMC1087172.**
28. Lambert, J. E., Ramos-Roman, M. A., Browning, J. D., & Parks, E. J. (2014). Increased de novo lipogenesis is a distinct characteristic of individuals with nonalcoholic fatty liver disease. *Gastroenterology*, 146(3), 726-735. doi:10.1053/j.gastro.2013.11.049. **PMC6276362.**
29. Schwarz, J. M., Linfoot, P., Dare, D., & Aghajanian, K. (2003). Hepatic de novo lipogenesis in normoinsulinemic and hyperinsulinemic subjects consuming high-fat, low-carbohydrate and low-fat, high-carbohydrate isoenergetic diets. *Am J Clin Nutr*, 77(1), 43-50. doi:10.1093/ajcn/77.1.43.
30. Blair, S. N. (1984). How to assess exercise habits and physical fitness. In J. D. Matarazzo, N. E. Miller, & S. M. Weiss (Eds.), *Behavioural health: A handbook of healthenhancement and disease prevention* (pp. 424-447). New York: Wiley.
31. NIAAA. (2003, October 15 and 16). The Task Force on Recommended Alcohol Questions: Recommended alcohol questions. Retrieved from <https://www.niaaa.nih.gov/research/guidelines-and-resources/recommended-alcohol-questions>
32. Tinker, L. F., Schneeman, B. O., & Willits, N. H. (1993). Number of weeks of 24-hour food records needed to estimate nutrient intake during a community-based clinical nutrition trial. *J Am Diet Assoc*, 93(3), 332-333. doi:10.1016/0002-8223(93)91564-7.
33. Santoro, N., Caprio, S., Pierpont, B., Van Name, M., Savoye, M., & Parks, E. J. (2015). Hepatic de novo lipogenesis in obese youth is modulated by a common variant in the GCKR gene. *J Clin Endocrinol Metab*, 100(8), E1125-1132. doi:10.1210/jc.2015-1587. **PMC4524990.**
34. Pineda, N., Sharma, P., Xu, Q., Hu, X., Vos, M., & Martin, D. R. (2009). Measurement of hepatic lipid: high-speed T2-corrected multiecho

- acquisition at 1H MR spectroscopy--a rapid and accurate technique. *Radiology*, 252(2), 568-576. doi:10.1148/radiol.2523082084.
35. Jequier, E., Acheson, K., & Schutz, Y. (1987). Assessment of energy expenditure and fuel utilization in man. *Annu Rev Nutr*, 7, 187-208. doi:10.1146/annurev.nu.07.070187.001155.
 36. Barrows, B. R., & Parks, E. J. (2006). Contributions of different fatty acid sources to very low-density lipoprotein-triacylglycerol in the fasted and fed states. *J Clin Endocrinol Metab*, 91(4), 1446-1452. doi:10.1210/jc.2005-1709.
 37. Hellerstein, M. K., & Neese, R. A. (1992). Mass isotopomer distribution analysis: a technique for measuring biosynthesis and turnover of polymers. *Am J Physiol*, 263(5 Pt 1), E988-1001. doi:10.1152/ajpendo.1992.263.5.E988.
 38. Parks, E. J., Skokan, L. E., Timlin, M. T., & Dingfelder, C. S. (2008). Dietary sugars stimulate fatty acid synthesis in adults. *J Nutr*, 138(6), 1039-1046. doi:10.1093/jn/138.6.1039. **PMC2546703**.
 39. Harris, J. A., & Benedict, F. G. (1918). A Biometric Study of Human Basal Metabolism. *Proc Natl Acad Sci U S A*, 4(12), 370-373. doi:10.1073/pnas.4.12.370. **PMC1091498**.
 40. Angulo, P., Hui, J. M., Marchesini, G., Bugianesi, E., George, J., Farrell, G. C., Enders, F., Saksena, S., Burt, A. D., Bida, J. P., Lindor, K., Sanderson, S. O., Lenzi, M., Adams, L. A., Kench, J., Therneau, T. M., & Day, C. P. (2007). The NAFLD fibrosis score: a noninvasive system that identifies liver fibrosis in patients with NAFLD. *Hepatology*, 45(4), 846-854. doi:10.1002/hep.21496.
 41. Vallet-Pichard, A., Mallet, V., Nalpas, B., Verkarre, V., Nalpas, A., Dhalluin-Venier, V., Fontaine, H., & Pol, S. (2007). FIB-4: an inexpensive and accurate marker of fibrosis in HCV infection. comparison with liver biopsy and fibrotest. *Hepatology*, 46(1), 32-36. doi:10.1002/hep.21669.
 42. Taylor, K. G., Carter, T. J., Valente, A. J., Wright, A. D., Smith, J. H., & Matthews, K. A. (1981). Sex differences in the relationships between obesity, alcohol consumption and cigarette smoking and serum lipid and apolipoprotein concentrations in a normal population. *Atherosclerosis*, 38(1-2), 11-18. doi:10.1016/0021-9150(81)90098-8.

APPENDIX A - NAFLD ACTIVITY SCORE SYSTEM

NAS grading sheet

Collection date:



Accession:

Biopsy Number:

Subject Name:

TORS Liver Pathology (NASH Clinical Research Network)

NAFLD Activity Score (NAS)

Steatosis (0-3)

- (0) <5%
- (1) 5%-33%
- (2) >33%-66%
- (3) >66%

Lobular Inflammation (0-3)

- (0) No foci
- (1) <2 foci per 200X field
- (2) 2-4 foci per 200X field
- (3) >4 foci per 200X field

Hepatocellular Ballooning (0-2)

- (0) None
- (1) Few balloon cells
- (2) Many cells/prominent ballooning

Unweighted sum of scores (0-8)

- ≥5 correlates with NASH
- ≤2 correlates with "not NASH"

NAFLD Staging

Fibrosis (0-4)

- (0) None
- (1) Perisinusoidal or periportal
 - (1A) Mild, zone 3, perisinusoidal (delicate fibrosis)
 - (1B) Moderate, zone 3, perisinusoidal (dense fibrosis)
 - (1C) Portal/periportal fibrosis (without perisinusoidal fibrosis)
- (2) Perisinusoidal and portal/periportal
- (3) Bridging fibrosis
- (4) Cirrhosis

Additional features:

Steatosis location (predominant distribution pattern)
(0 = Zone 3, 1 = Zone 1, 2 = azonal, 3 = panacinar)

Acidophil bodies (0 = none to rare, 1 = many)

Microvesicular steatosis (contiguous patches)

Pigmented macrophages
(0 = none to rare, 1 = many)

(0 = not present, 1 = present)

Megamitochondria (0 = none to rare, 1 = many)

Microgranulomas (0 = absent, 1 = present)

Mallory hyaline

Large lipogranulomas (0 = absent, 1 = present)

(0 = none to rare, 1 = many)

Portal inflammation

Glycogenated hepatocyte nuclei

(0 = none to minimal, 1 = greater than minimal)

(0 = none to rare, 1 = many)

Hepatocellular Dysplasia (0 = absent, 1 = present)

, if present:

Iron hepatocyte (0-4)

Endothelial Fe (0 = absent, 1 = present)

RE Iron (0 = none, 1 = few (Kupffer cells), 2 = majority)

TORS Liver Pathology (Ishak System)

Necroinflammatory Scores

A. Periportal or periseptal interface hepatitis (piecemeal necrosis) (0-4)

- (0) Absent
- (1) Mild (focal, few portal areas)
- (2) Mild/moderate (focal, most portal areas)
- (3) Moderate (continuous around <50% of tracts or septa)
- (4) Severe (continuous around >50% of tracts or septa)

B. Confluent necrosis (0-6)

- (0) Absent
- (1) Focal confluent necrosis
- (2) Zone 3 necrosis in some areas
- (3) Zone 3 necrosis in most areas
- (4) Zone 3 necrosis + occasional portal-central (P-C) bridging
- (5) Zone 3 necrosis + multiple (P-C) bridging
- (6) Panacinar or multiacinar necrosis

C. Focal (spotty) lytic necrosis, apoptosis and focal inflammation (0-4)

- (0) Absent
- (1) One focus or less per 10X objective
- (2) Two to four foci per 10X objective
- (3) Five to ten foci per 10X objective
- (4) More than ten foci per 10X objective

D. Portal inflammation (0-4)

- (0) None
- (1) Mild, some or all portal areas
- (2) Moderate, some or all portal areas
- (3) Moderate/marked, all portal areas
- (4) Marked, all portal areas

Total score for grading (0-18)

Ishak Stage (0-6)

Additional observations:
Pathologist signature: Diaz-Arias, Alberto A

APPENDIX B - INFORMED CONSENT FORMS

Chapter II - Consent form

CONSENT FORM TO PARTICIPATE IN A RESEARCH STUDY UNIVERSITY OF MISSOURI

INVESTIGATORS' NAMES: Majid M. Syed Abdul, MS, Elizabeth J Parks, PhD

STUDY STAFF: Jennifer Snawder, LD, RD

PROJECT #: 2012544

STUDY TITLE: "Contribution of de novo lipogenesis in severity of nonalcoholic fatty liver disease"

INTRODUCTION

We invite you to take part in this research study. This consent form tells you why we are doing the study, what will happen if you join the study, and other important information about the study. Please take as much time as you need to read this consent form. You can discuss it with your family, friends, or personal doctor. If there is anything you do not understand, please ask us to explain. Then you can decide if you want to take part in the study or not.

The Principal Investigators are Majid Syed-Abdul, MS and Elizabeth Parks, PhD. The people working with Majid on this study are called the study team. The U.S. National Institute of Health, American Society for Nutrition, and The University of Missouri (called the sponsor in this form) are paying for the study.

WHAT SHOULD I KNOW BEFORE I DECIDE WHETHER TO TAKE PART IN THIS STUDY?

Research helps us to learn new things and test new ideas about treating certain conditions/diseases. Taking part in research is voluntary. You decide if you want to take part, and you can stop taking part at any time. Your regular medical care at the University of Missouri Hospitals and Clinics will not be affected now or in the future if you decide you do not want to be in this study.

You are being asked to take part in this study because you are scheduled for bariatric surgery. As with any research study, there are risks that we know about and there may be some we don't know about. We will explain these risks in this form. We will only include you in this study if you give us your permission first by signing this consent form.

This study is being sponsored by the U.S. National Institute of Health, American Society for Nutrition, and The University of Missouri. In order to participate in this study, it will be necessary to give your written consent. About 100 people undergoing bariatric surgery will take part in this study.

WHY ARE THE RESEARCHERS DOING THIS STUDY?

The goal of this study is to test how excess body weight affects liver health. The tests performed are not part of standard medical care.

WHAT WILL HAPPEN IF I TAKE PART IN THIS STUDY AND HOW LONG WILL I BE IN THE STUDY?

If you decide to join this study, you will sign this form today and then, on the day of your surgery, you will undergo three procedures. Your participation will involve no additional time besides that time.

PROCEDURES

CONSENTING VISIT

During today's surgery orientation, this research is being presented. We will explain the study in detail; please ask any questions you might have.

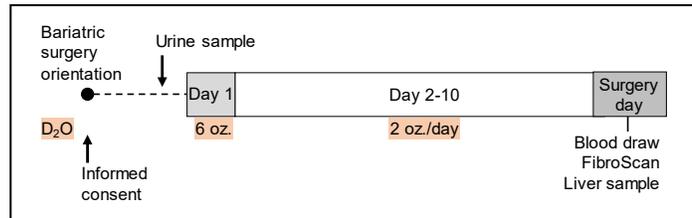
If you participate in this study, you will perform five activities

1. PRE-SURGERY RESEARCH

WATER CONSUMPTION

Our staff will contact you and provide you with special water which you will consume each day for 10 days before your surgery. This water is called

'heavy water' and your body will use it just like all the water in your diet. There are no risks to drinking the heavy water. If your body is normally making fat, this water will get incorporated into those fats. We can then determine how much fat was made in the last 10 days.



HOW TO CONSUME THE HEAVE WATER

You will drink a small amount of heavy water each day. On day 1, you will consume three small bottles of water and for the remaining days you will drink one bottle. Each bottle is labeled with the time and date you will need to drink it. You can drink this water with meals or by itself. There is no known risk associated with consumption of this water.

2. URINE SAMPLE

You will collect urine sample before starting the consumption of heavy water which will be used to measure the enrichment of D₂O in your body. You will collect one sample (1 ml) of their urine and store it in a small container provided by the researchers. You will also be provided with the appropriate containers/freezer packs to store your urine sample. You will bring this sample to the researcher during your pre-surgery visit at the MU hospital.

3. FIBROSCAN

The morning of your surgery, you would undergo a test called a FibroScan® which is an ultrasound procedure used to measure liver stiffness. The test requires that you to lie still on your back while a technician places a wand on your skin over your ribs. There are no known risks associated with this test. The test is painless and takes 15 to 30 minutes.

4. BLOOD DRAW

On the day of your surgery, you will have an IV line placed in your arm as part of your medical care. As part of this study, a nurse will draw a small amount of blood (about 1 1/2 tablespoons) to be used for research purposes.

5. HAVE A SMALL PORTION OF YOUR LIVER ANALYZED

As part of your surgery, you would have a small sample of liver taken to determine your liver health. For this research, a piece of that sample will be analyzed to find out how much fat is in your liver. The researcher will analyze the liver and look for the heavy water marker. The goal is to determine how much new fat the liver has made recently.

WHAT ARE THE PROCEDURES AND RISKS OF BEING IN THIS STUDY?

The risks associated with being in this study include the fact that your health information will be entered into a data base. Your name will not be included in that data base. The following information will be included: medical record number, date of birth, sex, ethnicity, body weight, height, blood pressure, blood values of glucose, cholesterol, and hormones like insulin, and markers of liver health. The researchers will also record recent medication use and your family history of disease. Since you are already having an IV line put in your arm, taking some blood should not pose a risk to your health.

WHAT ARE THE HEALTH RISKS OR WHAT PROBLEMS CAN I EXPECT FROM THE STUDY?

The risk associated with being in the study is that your health information will be entered into a data base. Your name will not be included in that data base. The following information will be included: medical record number, date of birth, sex, ethnicity, body weight, height, blood pressure, blood values of glucose, cholesterol, and hormones like insulin, and markers of liver health.

The researchers will also record recent medication use and your family history of disease. Since you are already having an IV line put in your arm, the risks include taking 2 tablespoons of blood and the harvest of a small amount of fat from your belly. This small amount of blood should not pose a risk to your health. The risk of donating fat and liver tissue is some bleeding.

ARE THERE BENEFITS TO TAKING PART IN THE STUDY?

If you agree to take part in this study, there will be no direct benefit to you. We hope the information will help us to learn more about how body weight affects liver disease in the future.

WHAT OTHER CHOICES DO I HAVE?

You do not have to participate in this study.

WHAT ABOUT PRIVACY AND CONFIDENTIALITY?

Information produced by this study will be stored in the investigator's file and identified by a code number only. The code key connecting your name to specific information about you will be kept in a separate, secure location. Your data will be shared and added to that of a larger study in bariatric surgery patients being conducted by Dr. Elizabeth Parks. Information contained in your records may not be given to anyone unaffiliated with the study in a form that could identify you without your written consent, except as required by law. It is possible that your medical and/or research record, including identifying information, may be inspected and/or copied by the study sponsor (and/or its agent), federal or state government agencies, MU Health Sciences IRB, or hospital accrediting agencies, in the course of carrying out their duties. If your record is inspected or copied by the study sponsor (and/or its agents), or by any of these agencies, the University of Missouri will use reasonable efforts to protect your privacy and the confidentiality of your medical information.

The results of this study may be published in a medical journal or used for teaching purposes. However, your name or other identifying information will not be used in any publication or teaching materials without your specific permission.

AUTHORIZATION FOR THE USE AND DISCLOSURE OF PERSONAL HEALTH INFORMATION RESULTING FROM PARTICIPATION IN A RESEARCH STUDY

State and federal privacy laws (HIPAA) protect the use and release of your health information. If you decide to take part in this study, you also give us your permission to use your private health information, including the health information in your medical records and information that can identify you. You have the right to refuse to give us your permission for us to use your health information. However, doing so would mean that you could not take part in this study.

The following identifiers and information will be obtained from your health records:

- ✓ Medical Record Number
- ✓ Birthdate
- ✓ Demographics (age, race) mass index (BMI)
- ✓ Smoking history
- ✓ Progress notes
- ✓ Pathology reports
- ✓ Contact information such as address, phone number
- ✓ Consultations information such as age, race etc.
- ✓ Name
- ✓ Date of surgery
- ✓ Body weight and body
- ✓ Medications
- ✓ History and physical exams
- ✓ Laboratory reports
- ✓ Demographic

We may share any of this information with the following

- Authorized members and staff of the University of Missouri Institutional Review Board (IRB),
- Study monitors or auditors who make sure that the study is being done properly,
- Government agencies and public health authorities such as the Department of Health and Human Services (DHHS) and the Office of Human Research Protections (OHRP).

Any research information shared with outside entities will not contain your name, address, telephone or social security number, or any other personal identifier unless it is necessary for review or required by law. The people who get your health information may not be required by Federal privacy laws (such as the HIPAA Privacy Rule) to protect it. Some of those people may be able to share your information with others without your separate permission. The University of Missouri will use reasonable efforts to protect your privacy and the confidentiality of your medical information. Your permission for us to use and/or release your information will not expire unless you cancel your permission.

You can cancel your permission at any time by writing to:

Dr. Elizabeth Parks or Majid Syed-Abdul

University of Missouri Department of Nutrition and Exercise Physiology

Room NW406, One Hospital Drive, Columbia, MO 65212

You have the right to access your protected health information that is obtained or created during this research project until the end of study ends. If you have not already received a copy of the University of Missouri Healthcare Privacy Notice, you may request one. If you have any questions or concerns about your privacy rights, you may contact the Privacy Officer at 573-882-9054.

CAN I SEE MY RESEARCH RECORDS?

If you wish to have a copy of your results, please let the researchers know how you would like to receive them (by email or in the mail).

ARE THERE ANY COSTS TO BEING IN THE STUDY?

The study will pay for all research tests and procedures. You and/or your health plan or insurance will not be billed for tests and procedures that are done in this research study.

WILL I BE PAID FOR PARTICIPATING IN THE STUDY?

You will be paid \$50 for your participation in this study which requires the consumption of the heavy water. You will be paid by a check, which is sent to you through the mail, which usually takes 1-2 weeks to arrive. We will need your social security number in order to pay you. Any payment may need to be reported as income on your tax return. If you are not a resident/ citizen (non-resident alien) of the United States, you will need to work with the MU Nonresident Tax Specialist at 573-882-5509.

WHAT HAPPENS IF I AM INJURED DURING THE STUDY?

It is not the policy of the University of Missouri to compensate human subjects in the event the research results in injury. The University of Missouri, in fulfilling its public responsibility, has provided medical, professional and general liability insurance coverage for any injury in the event such injury is caused by the negligence of the University of Missouri, its faculty and staff. The University of Missouri also will provide, within the limitations of the laws of the State of Missouri, facilities and medical attention to subjects who suffer injuries while participating in the research projects of the University of Missouri.

In the event you have suffered injury as the result of participation in this research program, you are to contact the Risk Management Officer, telephone number (573) 882-1181, at the Health Sciences Center, who can review the matter and provide further information. This statement is not to be construed as an admission of liability.

WHAT ARE MY RIGHTS AS A PARTICIPANT?

Participation in this study is voluntary. You do not have to participate in this study. Your present or future care will not be affected should you choose not to participate. If you decide to participate, you can change your mind and drop out of the study at any time without affecting your present or future care at the University of Missouri. Leaving the study will not result in any penalty or loss of benefits to which you are entitled. In addition, the investigators of this study may decide to end your participation in this study at any time. You will be informed of any significant new findings discovered during the course of this study that might influence your health, welfare, or willingness to continue participation in this study.

WHERE CAN I GET MORE INFORMATION ABOUT THIS STUDY?

A description of this clinical trial is available on www.ClinicalTrials.gov, as required by U.S. law. This Website will not include information that can identify you. At most, the Web site will include a summary of the results. You can search this Website at any time by entering this study's number: NCT03683589.

WHO CAN ANSWER MY QUESTIONS ABOUT THE STUDY?

If you have more questions about this study at any time, you can call Dr. Parks at (682) 433-9012. You may also contact the University of Missouri Institutional Review Board if you

- Have any questions about your rights as a study participant,
- Want to report any problems or complaints, or,
- Feel under any pressure to take part or stay in this study.

The IRB which is a group of people who review the research studies to make sure the rights of participants are protected. Their number is (573) 882-3181. If you want to talk privately about your rights or any issues related to your participation, you can contact the University of Missouri Research Participant Advocate (RPA) at (888) 280-5002 (a free call), or emailing

MUResearchRPA@missouri.edu. We will give you a copy of this consent form. Please keep it where you can find it easily. It will help you remember what we discussed today.

CONSENT TO PARTICIPATE

By signing my name below, I confirm the following:

- I have read or had read to me this entire consent form.
- All of my questions were answered to my satisfaction.
- The study's purpose, procedures, risks and possible benefits were explained to me.
- I voluntarily agree to take part in this research study. I have been told that I can stop at any time.

Subject name (print)	Subject signature	Date	Time
----------------------	-------------------	------	------

Staff obtaining consent (print)	Staff signature	Date	Time
---------------------------------	-----------------	------	------

Chapter III - Consent form

CONSENT FORM TO PARTICIPATE IN A RESEARCH STUDY

INVESTIGATORS' NAMES: Camila Manrique, MD, Ghassan Hammoud, MD,
and

Elizabeth J Parks, PhD, at the University of Missouri
in Columbia, MO

STUDY STAFF: Kimberlee Bingham, BS Majid Syed-Abdul, MS
Nathan Le, BS

MU PROJECT #: 2006432 **CLINICALTRIALS.GOV NUMBER:** NCT02948569

STUDY TITLE: "Evaluation of TVB-2640, a FASN Inhibitor, to reduce de novo lipogenesis in subjects with characteristics of the metabolic syndrome"

INTRODUCTION

This consent may contain words that you do not understand. Please ask the investigator or the study staff to explain any words or information that you do not clearly understand. This project is a research study. Research studies include only people who choose to participate. As a study participant you have the right to know about the procedures that will be used in this research study so that you can make the decision whether or not to participate. The information presented here is simply an effort to make you better informed so that you may give or withhold your consent to participate in this research study. Please take your time to make your decision. This study is being sponsored by the Company, 3V Biosciences, who has developed an investigational drug to treat liver disease in overweight people. This drug is not approved by the FDA at this time. One of the Co-Investigators, Elizabeth Parks, consults for this company and therefore she received financial compensation from the company sponsoring this research. Elizabeth Parks has designed the study and a physician, Dr. Manrique will be conducting it.

What is this study about? People who are overweight can begin to store fat in their liver. This fat is made from dietary sugar and can cause the liver to not work well. You are being asked to take part in this study because your body weight increases the likelihood that your liver will make fat. In order to participate in this study, it will be necessary to give your written consent.

The following definitions may help you understand this study:

- **“Researchers”** means the study doctor and research personnel at the University of Missouri.
- **“Stable isotopes”** are naturally occurring elements used to help us trace sources of fat in the blood. Stable isotopes are not radioactive.

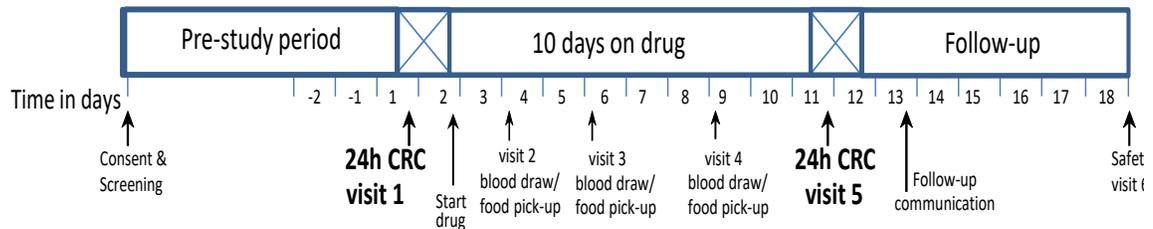
WHY IS THIS STUDY BEING DONE AND HOW MANY PEOPLE WILL TAKE PART?

The company 3V Biosciences has developed an investigational drug called TVB-2640. This drug is not approved by the FDA at this time. In preliminary studies, this drug has been shown to lower liver fat production but the ideal dose for its use has not been decided. The purpose of this study is to test the drug in overweight men, to determine the appropriate dose. The information from this study will enable future studies to test the drug's effects in patients with liver fat due to disease. The tests performed are not part of your standard medical care. Up to 100 people will be screened to find the 12 people who will take part in this study.

WHAT IS INVOLVED IN THE STUDY AND HOW LONG WILL I PARTICIPATE?

This research is measuring how the drug may improve liver health. You will be screened to find out if you are eligible to participate. As shown below, over 20-day period this research includes 2 inpatient study visits, 4 safety blood draws, and a daily dose of the drug for 10 days.

Study Plan



STUDY TIMELINE

The following paragraphs describe the schedule for the study screening visit and five study visits.

SCREENING VISIT

To help decide if you qualify to be in this study, the researchers will ask you questions about your health, including medications you take and any surgeries you have had. This visit is located at the Clinical Research Center (CRC). The CRC is on the 5th floor of the University Hospital and has clinic rooms specially designed for research. You will come to the CRC after fasting overnight for 12 hours (no food or drink, except water). This visit will take 1 hour and you are welcome to bring a family member or close friend to this visit.

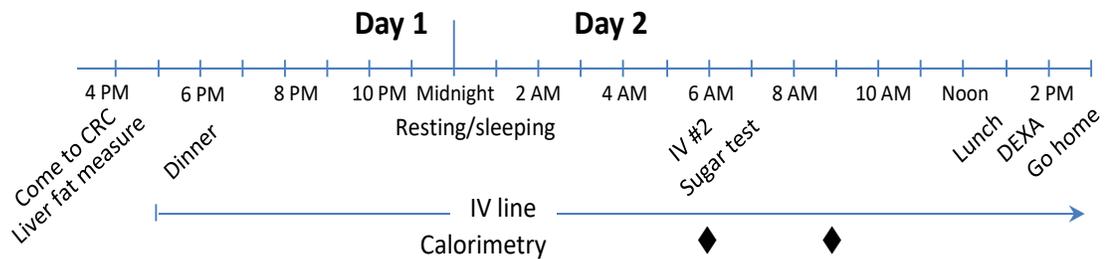
Research staff will meet with you to review the study procedures, answer questions, sign this consent form and review your medical history. A small amount of blood will be drawn (about 2.5 tablespoons) to check your general health which includes a test for hepatitis as well as a measurement of your fasting blood sugar to ensure you are not diabetic. Your urine will be tested for drugs. Your height, weight, and blood pressure will be measured and you will be given a chance to learn about a procedure called calorimetry, which we use to

measure how many calories your body burns. If you enter the study, this technique will be performed four times. Within a week after the screening visit, you will receive the results of all screening tests and someone on the study team will go over the results with you. Since this is not a treatment study, these lab tests are not being used for standard medical care. You will be given a copy of the results which you can share with your primary physician.

If your screening results qualify you to be in the study, and if you are interested in proceeding with the study, you will be scheduled for a second, baseline visit. Again, you will come to the CRC at the hospital and parking will be provided if needed. For 3 days before this baseline visit, you will be provided a 3-day controlled diet which is made fresh and based on foods you normally prefer. You must consume only these meals, all of the food provided, and no other food. You may not consume alcohol between days -2 and 13 of the study.

BASELINE VISIT 1: On the day of your first overnight hospital test, you will come into the CRC at 4:00 PM. This visit will take about 24 hours and you will undergo several procedures. The first procedures include assessments of your liver health. This will include Magnetic Resonance Imaging (MRI) and also, potentially, a procedure called the FibroScan. These, and all the procedures are described in detail below. When you return to the CRC after your scan, an IV line will be placed in a vein in one of your arms. Next, an IV solution is infused. This solution is called an isotopic tracer and it will continue until 2 PM the next day. You will be fed dinner and then undergo a skin test in which a piece of tape will be placed on your forehead for 30 minutes to collect a sample of the oils produced by your skin.

Overnight hospital test



You will then sleep overnight in the CRC. At 6 AM the next morning, you will arise and wash up. Then, a second IV line is placed in your other arm and this one will be used to draw blood. You will undergo a procedure called calorimetry, which measures the amount of calories you burn. You will then consume a sugary drink and blood will be drawn off and on until 2 PM. You will undergo calorimetry a second time. After the final blood draw, you will be fed lunch and undergo a procedure called a DEXA scan. The DEXA scan measures your body composition. After this you will be discharged and can go home.

If all baseline procedures are successful, you will begin the drug treatment regimen for the next 10 days. You will take a single daily dose of the drug orally before bedtime or 10:30 PM, whichever comes first. The physician assigned to this study will oversee the prescribed dosage you are instructed to take.

SAFETY BLOOD DRAW VISIT 2: The morning of this visit, please do not exercise, and for 12 hours before the visit, do not use caffeine and vitamin supplements. You will come into the CRC after fasting overnight for 12 hours - no food or drink, except water after 7 PM. A small amount of blood will be taken (about 1 tablespoon) and we will check your weight, blood pressure, heart rate, and temperature. This visit will take about 30 minutes.

SAFETY BLOOD DRAW VISITS 3: This visit is the same as visit #2 above. A small amount of blood will be taken (about 1 tablespoon).

SAFETY BLOOD DRAW VISITS 4: This visit is the same as visit #2 above with the addition of a collection of skin oils. A piece of tape will be placed on your head for 30 minutes to collect the oils produced by your skin. The morning of this visit, do not apply any lotion or skin products on your face. A small amount of blood will be taken (about 1 tablespoon). This visit will take about 1 hour.

FOLLOW-UP VISIT 5: Just as you did for the baseline visit, you will come in to the CRC at 4 PM and the procedures will be the same. However, in this visit, blood will also be drawn 4 times during the night. This visit will take 24 hours and the total blood draw will be about 2 oz or about 2 tablespoons. Following this visit, the treatment portion of this study is over and you will discontinue taking the drug.

SAFETY BLOOD DRAW VISIT 6: Approximately 5-7 days following visit 5, you will come into the CRC for final safety blood draw (1 tablespoon). The same procedures will be done at this visit as with safety visits 2 and 3. This visit will take about 45 minutes.

WHAT ARE THE PROCEDURES AND RISKS OF BEING IN THIS STUDY?

STUDY DRUG

The study drug, TVB-2640, has been given in preliminary studies up to 2 months in duration. Side effects were seen in some subjects as early as 10 days on the drug and included dry and sore eyes, some hair loss, and upset stomach. Also, dry skin on the hands and feet which can also feel sore. All side effects went away after the subjects stopped taking the drug. These side effects were seen at higher doses than will be used here, and when the drug was given for longer durations. In the present study, you will take the drug for 10 days. You will be monitored closely and blood samples will be taken every couple of days to document the drug's effects. If you notice any side effects such as dry skin, nausea, or eye problems, please notify the study staff immediately. If your skin gets dry you will be provided with lotion to reduce this side effect. Additionally, it is unknown whether this drug has any effects

on a man's ability to reproduce. We advise all subjects to use contraception (condoms) during the duration of this study.

If you experience side effects the following may occur. First, we would ask to take a picture of your hands if you have dry skin, or of the top of your head if you had some hair loss. You would need to sign a separate release form for this photography. The photo would not show your face or reveal your identity in any way. Second, if side effects are present during safety visit 6, you will be asked to return to the CRC every 2 weeks for up to 3 additional visits (two weeks, four weeks, and six weeks after your last dose of drug). On your first follow-up visit (two weeks post-drug), the Sebutape test will be repeated. There is no additional risks for repeating this test.

STUDY DIET: FOOD AND BEVERAGES

If you participate in the study you will be provided all your food during the 10 days you are taking the drug. This food is commercially available (bought from HyVee) and the meals are made both fresh and also made up of some frozen meals. When you come in for the safety blood draws, you will be given the food in a cooler to take home. If you prefer, you may be provided a couple of cans of soda to drink during this time (no other soda than these can be consumed). Otherwise, only water, tea, and coffee are allowed (cream and any kind of sweetener is allowed). No additional milk shakes, protein shakes, or energy drinks should be consumed during this study.

It is advised that if you have a special event coming up in the next month (wedding, vacation travel, etc.) that you not participate in the study at this time. You should not consciously change your physical activity while you are in the study. In other words, if you would like to join a gym or begin exercising more, we ask that you wait until the study is over before you do this.

CALORIMETRY

This test measures how many calories your body uses. This procedure will be performed at visit 1 and visit 5. Calorimetry requires resting quietly on your back for 20-30 minutes under a large, clear, plastic hood. You will breathe room air normally and your breath goes into an analyzer to measure what you breathe out. The test is painless; however, persons who are uncomfortable in confined spaces may find this slightly stressful. You will get a chance to familiarize yourself with this procedure during screening.

MEASUREMENT OF BODY FAT AND MUSCLE BY DEXA SCAN

This test will be performed at visit 1 and 5. A DEXA (dual energy x-ray absorptiometry) scan is a procedure to measure your body composition - how much fat and muscle your body has. It is a type of x-ray machine with a moving arm. This procedure involves lying on a table for 20-30 minutes while the DEXA machine passes over your body. Although you will need to remain very still and quiet, you will feel nothing and should have no discomfort. If you have participated in any other research study involving ionizing radiation exposure (x-rays) in the past 12 months, discuss this with the Investigator to determine if you

are eligible to participate in this study. You will be exposed to a small radiation dose which is about 2% of the average radiation dose from all sources (natural background radiation, consumer appliances, radon gas, medical tests, etc.) that a person receives in the United States receives each year. However, radiation effects add up. If you need an x-ray in the next year, you should inform your doctor of your participation in this study.

MEASUREMENT OF LIVER FAT BY MRI

This test will be performed at visit 1 and 5 at either the department of Radiology at University Hospital or at the Missouri Imaging Center at South Providence Medical Park. Transportation will be provided. MRI (magnetic resonance imaging) is a technique used to measure the amount of fat present in your liver. The risks of undergoing an MRI could include some psychological stress from a banging noise during the scan. Subjects may experience nervousness from confinement in a tight space (claustrophobia). Magnetic fields to be used in this study have no known hazards to human subjects whose bodies do not contain any magnetic metals (see below for protection from risks). If you feel anxious, you can stop the procedure at any time. You may experience some discomfort and fatigue from lying still during scanning. You will be questioned using a standardized interview about whether you have a heart pacemaker, metallic objects, vascular clips, electrodes, cochlear implants, neurostimulators, shunts, heart valve implants, penile implants, vascular filters, rods & screws, post CABG pacer wires, colored contact lens, dental prostheses, limb prostheses, eye prostheses, shrapnel, metal in head, eye, or skin, and embolization coils. If such devices cannot be removed safely, you will be excluded from the study. We have a mock scanner to allow you to practice the procedure. When the scan is occurring, the radiology technician is in constant contact with you through head phones.

FIBROSCAN

This procedure is designed to measure the stiffness of your liver. It requires that you lie still on your back while a technician places a wand on your skin over your ribs. The test is painless and takes 15 to 30 minutes.

SKIN OIL COLLECTION

One of the side effects of the drug can be dry skin. To monitor this side effect, a small sample of skin oils will be collected from your forehead using a piece of tape that is placed on the skin for 30 minutes. There are no risks associated with this procedure.

RISKS OF BLOOD DRAWING

During screening and at visits 1-5, you will have blood drawn through a needle. Risks associated with drawing blood from your arm include minimal discomfort and/or bruising. Infection, excess bleeding, clotting, and/or fainting also are possible, although unlikely. As a result of your participation in this study you will have donated blood. If you wish to participate in other research after you finish

this project, you should let the investigator know that you have given about 6 oz (or about 3/4 cup). Your blood volume will be checked during screening to make sure that it is in safe limits.

INTRAVENOUS CATHETERIZATION

During baseline visit 1 and follow up visit 5, you will have an IV line placed that is identical to the IV that a person receives when admitted to the hospital. While IVs are normally safe and painless, there is always a very small risk of infection, bleeding, or bruising associated with the catheter. Should the IV fall out of the vein, it is possible that some of the liquid will go into the skin rather than the vein. This may result in swelling and pain around the area of the IV, and the IV would have to be placed in a different location on the arm or hand. The IV lines will be in for about 20 hours. The protocols that the CRC nurses follow to reduce risk of infection include: cleaning the skin thoroughly before IV placement, securing the IV with tape, covering the site with clean gauze, and protecting the site using an elastic sleeve. The site is inspected at each blood draw – with particular attention paid to any changes in the surrounding skin.

HOSPITAL STAYS

During your visit you will be restricted to the CRC. Although quiet leisure activities can be provided, confinement to the CRC may be boring or you may feel hungry. You may move about your assigned hospital room and use the facilities as needed, unless the test being performed requires bed-rest. Some people may find it hard to be confined to a hospital bed for the duration of the test. For the reasons stated above the investigator will observe you closely while giving the treatment described and, if you have any worrisome symptoms or symptoms that the investigator or her associates have described to you, notify the investigator immediately. Investigator's telephone number is (573) 529-1141. For more information about risks and side effects, ask the investigator or contact study coordinator, Kimberlee Bingham at 573-884-1708.

WHAT WILL BE MY RESPONSIBILITIES DURING THE STUDY AND ARE THERE BENEFITS?

While you are part of this study, the researchers will follow you closely to determine whether there are problems. It is your responsibility to do the following:

- Ask questions about anything you do not understand.
- Keep your appointments.
- Follow the researchers' instructions, particularly in consuming the medication.
- Let the researchers know if your telephone number or address changes.
- Tell the researchers before you take any new medication, even if it is prescribed by another doctor for a different medical problem, or purchased over the counter.
- Report to the researchers any injuries or illnesses while you are on the study, even if you do not think they are related.

If you agree to take part in this study, there will be no direct medical benefit to you. You may expect to benefit from taking part in this research to the extent that you are contributing to medical knowledge. You will receive the results of all of your tests. We hope the information learned will benefit others in the future.

WHAT ARE THE COSTS?

You will not be charged for any procedures that are part of this research study. Parking will be provided but there is no compensation for travel to our facilities or for childcare during this study.

WILL I BE PAID FOR PARTICIPATING IN THE STUDY?

You will be compensated a total of \$1,200 for completing this study as follows: \$150 will be given for baseline visit 1; \$75 will be given for safety visit 2, \$100 for safety visit 3 and \$150 for visit 4, \$225 for consuming the 14-day diet and completing follow-up visit 5, and \$500 for the final safety blood draw.

If you experience side effects, you will be asked to return to the CRC up to three times over six weeks. If this occurs, you will be paid an additional \$25 per visit.

WHAT OTHER OPTIONS ARE THERE?

You do not have to participate in this study.

WHAT ABOUT CONFIDENTIALITY?

Information produced by this study will be stored in the investigator's file and identified by a code number only. The code key connecting your name to specific information about you will be kept in a separate, secure location. Information contained in your records may not be given to anyone unaffiliated with the study in a form that could identify you without your written consent, except as required by law.

It is possible that your medical and/or research record, including identifying information, may be inspected and/or copied by the study sponsor (and/or its agent), the Food and Drug Administration (FDA), federal or state government agencies, MU Health Sciences IRB, or hospital accrediting agencies, in the course of carrying out their duties. If your record is inspected or copied by the study sponsor (and/or its agents), or by any of these agencies, the University of Missouri will use reasonable efforts to protect your privacy and the confidentiality of your medical information. The results of this study may be published in a medical journal or used for teaching purposes. However, your name or other identifying information will not be used in any publication or teaching materials without your specific permission.

WHAT IF I AM INJURED?

In the event the research results in injury, the Sponsor agrees that it, and not Institution, will be responsible for the costs of diagnosis, care and treatment of any undesirable side effects, adverse reactions, illness or injury. The study staff will coordinate your care, should you need it. In the event you have suffered injury

as the result of participation in this research program, you are to contact the Risk Management Officer, telephone number (573) 882-1181, at the Health Sciences Center, who can review the matter and provide further information.

WHAT ARE MY RIGHTS AS A PARTICIPANT?

Participation in this study is voluntary. You do not have to participate in this study. Your present or future care will not be affected should you choose not to participate. If you decide to participate, you can change your mind and drop out of the study at any time without affecting your present or future care at the University of Missouri. Leaving the study will not result in any penalty or loss of benefits to which you are entitled. In addition, the investigator of this study may decide to end your participation in this study at any time after she has explained the reasons for doing so and has helped arrange for your continued care by your own doctor, if needed. You will be informed of any significant new findings discovered during the course of this study that might influence your health, welfare, or willingness to continue participation in this study.

The Data Safety Monitor for this study is Dr. Ghassan Hammoud, a liver expert. He will be reviewing the blood draw values throughout the time you are participating to monitor your safety. Our study staff will tell you about the new information from this or other studies that may affect your health, welfare, or willingness to continue participation in this study. A description of this clinical trial is available on www.ClinicalTrials.gov, as required by U.S. law. This Web site will not include information that can identify you. At most, the Web site will include a summary of the results. You can search this Web site at any time.

WHOM DO I CALL IF I HAVE QUESTIONS OR PROBLEMS?

If you have any questions regarding your rights as a participant in this research and/or concerns about the study, or if you feel under any pressure to enroll or to continue to participate in this study, you may contact the University of Missouri Institutional Review Board (which is a group of people who review the research studies to protect participants' rights) at (573) 882-3181. You may also contact the Research Participant Advocate (RPA) at (573) 884-1925 or (888) 280-5002 (toll-free). If you prefer email, you can reach the Advocate at somrpa@missouri.edu.

You may ask more questions about the study at any time. For questions about the study or a research-related side effects or injury, contact Dr. Camila Manrique at (573) 882-2273. A copy of this consent form will be given to you to keep.

SIGNATURES

I confirm that the purpose of the research, the study procedures, the possible risks and discomforts as well as potential benefits that I may experience have been explained to me. Alternatives to my participation in the study also have

been discussed. I have read this consent form and my questions have been answered.

My signature below indicates my willingness to participate in this study.

Subject name (print)	Subject signature	Date	Time
----------------------	-------------------	------	------

Staff obtaining consent (print)	Staff signature	Date	Time
---------------------------------	-----------------	------	------

Chapter IV - Consent form

CONSENT FORM TO PARTICIPATE IN A RESEARCH STUDY

INVESTIGATOR'S NAME: ELIZABETH PARKS, PHD

PROJECT # 1211233

THE TAILGATE STUDY: "The Tailgate Study: A pilot study measuring the impact of acute alcohol intake on intrahepatic lipid"

INTRODUCTION

This consent may contain words that you do not understand. Please ask the investigator or the study staff to explain any words or information that you do not clearly understand.

This is a research study. Research studies include only people who choose to participate. As a study participant you have the right to know about the procedures that will be used in this research study so that you can make the decision whether or not to participate. The information presented here is simply an effort to make you better informed so that you may give or withhold your consent to participate in this research study. **Please take your time to make your decision and discuss it with your family and friends.**

You are being asked to take part in this study because you responded to a flyer/advertisement and may meet the criteria to be in the study.

This study is being sponsored by The University of Missouri. In order to participate in this study, it will be necessary to give your written consent.

The following definitions may help you understand this study:

- **"Researchers"** means the study doctor and research personnel at the University of Missouri.
- **"Stable isotopes"** are naturally occurring elements used to help us trace sources of fat in the blood. Stable isotopes are not radioactive.

WHY IS THIS STUDY BEING DONE?

The purpose of this study is to help researchers better understand the impact of excess consumption of alcohol and fatty food on the liver's health.

HOW MANY PEOPLE WILL TAKE PART IN THE STUDY?

About 50 people will take part in this study at the University of Missouri.

PART 1. WHAT IS INVOLVED IN THE STUDY?

If you take part in this research study, you will have the following tests and procedures. As described below, this study has two parts: one screening visit

and one overnight hospital stay. The procedures are being done solely for the purpose of this study.

Screening Procedures

To help decide if you qualify to be in this study, the researchers will ask you questions about your health, including medications you take and any surgeries you have had, alcohol consumption and physical activity level. The visits for this study are located at the Clinical Research Center (CRC). The CRC has both a clinic and hospital rooms specially designed for research and all are located on the 5th floor of the University Hospital.

For screening visit, you will come to the CRC after fasting overnight for 12 hours (no food or drink, except water). This visit will take 1½ hours and you are welcome to bring a family member or close friend to this visit. Research staff will meet you at the CRC to review the study procedures, answer questions, sign this consent form, and review your medical history. You will also have blood drawn (about 2 tablespoons) and this blood will be tested to assess your general health. Your height, weight, and blood pressure will be measured. You will receive the results of all screening tests and someone on the study team will go over your screening results with you. Since this is not a treatment study, these lab tests are not being used for standard medical practice. In other words, the tests are not being done to look for abnormalities, but you will be given a copy of the results which you could share with your primary physician.

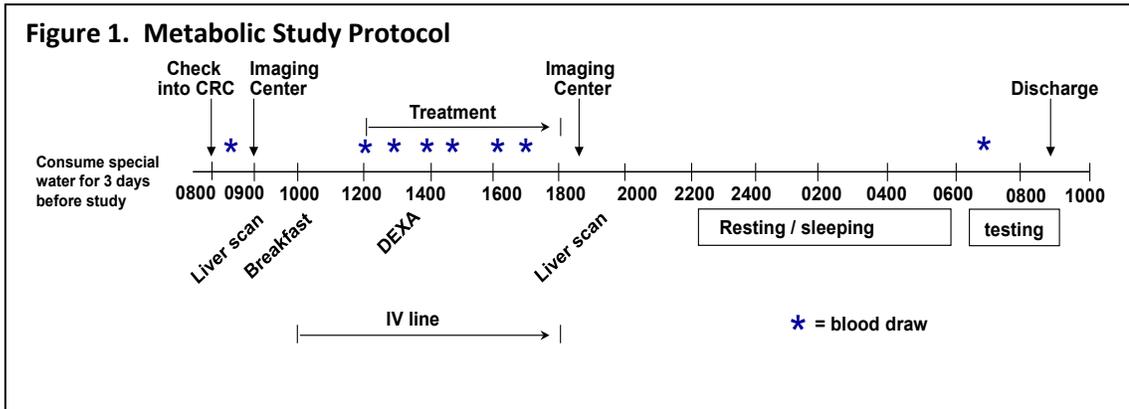
Pre-study diet

For three days before the overnight stay in the hospital, you will be advised on foods to consume. This diet will be based on your usual preferences and you will also receive small doses of a special kind of water, labeled with a cold (non-radioactive) isotope. This water is used to measure your liver's ability to make fat from the alcohol you consume when you are in the CRC. During the first two days of this pre-study diet, you may consume a single serving of alcohol, but no alcohol should be consumed on day 3 (the day before your inpatient study). Also, you should not exercise the day before the metabolic study.

The treatment day and in-patient hospital stay

The figure below shows the timing of your hospital activities. The CRC in-patient unit has typical hospital rooms and on the day of your study, you will report to the CRC at 8:00 AM. You will check into your room and your height and weight will be measured. An IV line will be placed in one of your arms and a small amount of blood drawn. You will then be transported to the Imaging Center where a scan of your liver will be taken. After this, you will be fed a light breakfast and brought back to the CRC. At noon, you will also be asked to consume repeated doses of an alcoholic beverage (vodka and juice). Your breath alcohol level will be monitored and you will likely become inebriated. At 12:30 PM, you will be offered a generous amount of food, consisting of typical tailgate fare (sausages, hotdogs,

burgers, etc.). You will be encouraged to consume the food and beverages in small doses consistently throughout the afternoon.



Timeline for Meal Test

At 5 PM, you will be transported back to the Imaging Center for a second liver scan. After this, you will return to the CRC where the IV line will be removed and you will sleep overnight. No additional alcohol will be consumed at this time but food will be available until 8:00 PM if you are hungry. The next morning, your breath will be tested for alcohol level, your metabolism assessed by a test that measures how much fat and glucose you are burning, and you will have a single small blood draw through a needle stick. After this, you will complete a survey to assess any symptoms of hangover. Finally, you will be given breakfast and allowed to leave the unit. Each of the activities that you will participate in is described in more detail below.

Test 1 – Liver Scan by Magnetic Resonance Spectroscopy (MRS)

This test will be performed in the Department of Radiology either in the Hospital or at the Missouri Radiology Imaging Center by qualified personnel and will occur on the morning and afternoon of your study. This test takes about 30-45 minutes and you will have to lie on a table which slides into the MRI machine. The test is painless; however, persons who are uncomfortable in confined spaces may find this test stressful.

Test 2 – Measurement of Body Fat by DEXA

This test will be performed on day 1 of the inpatient study. A DEXA (Dual Energy X-ray Absorptiometry) is a procedure to measure your body composition - how much fat and muscle your body has. It is a type of x-ray machine with a moving arm. This procedure involves lying on a table for 20-30 minutes while the DEXA machine passes over your body. Although you will need to remain very still and quiet, you will probably feel no discomfort.

Test 3 – Indirect Calorimetry

This test measures how many calories your body uses and how much fat and glucose you are burning. This procedure will be performed one time on the morning after your overnight stay in the CRC (7:00 AM). The test requires resting quietly on your back for 15 to 30 minutes under a large, clear, plastic

hood. You will breathe room air normally and your breath goes into an analyzer to measure the air that you breathe out. The test is painless; however, persons who are uncomfortable in confined spaces may find this test stressful.

Test 4 – Morning Assessment

The morning following your overnight stay, you will be asked to complete a brief questionnaire designed to measure symptoms related to hangover, such as headache, dizziness, nausea, and balance. The assessment is a paper and pencil test that will take approximately 10 minutes to complete.

HOW LONG WILL I BE IN THE STUDY?

You will be in this study for approximately 24 hours, however, you will have some participation for one screening visit and another three days where you will be asked to consume water labeled with stable isotopes. **You can stop participating at any time. Your decision to withdraw from the study will not affect in any way your medical care and/or benefits.**

~~~~~  
~~~~~

PART 2. What Are the Risks of the Study?

While on the study, you are at risk for the side effects described below. You should discuss these with the investigator and/or your doctor. There may also be other side effects that we cannot predict. Many side effects go away shortly after the intervention is stopped, but in some cases side effects can be serious or long-lasting or permanent.

Risks of Blood Drawing

Risks associated with drawing blood from your arm include minimal discomfort and/or bruising. Infection, excess bleeding, clotting, and/or fainting also are possible, although unlikely. You will have a little more than ½ cup of blood collected over several hours because you are in this research study. Because you have given blood, you should not participate in any other study while you are in this one and wait 8 weeks following completion of these tests before donating blood.

Intravenous Catheterization

During study day, you will have an intravenous (IV) line placed that is identical to the IV that a person receives when admitted to the hospital. While IVs are normally safe and painless, there is always a very small risk of infection, bleeding, or bruising associated with the catheter. Should the IV fall out of the vein, it is possible that some of the liquid will go into the skin rather than the vein. This may result in swelling and pain around the area of the IV, and the IV would have to be placed in a different location, arm or hand.

The IV lines will be in for 12 hours. The protocols that the CRC nurses follow to reduce risk of infection include: cleaning the skin thoroughly before IV placement,

securing the IV with tape, covering the site with clean gauze, and protecting the site using an elastic sleeve. The site is inspected at each change in nursing shift – with particular attention to any changes in the surrounding skin with respect to swelling, color, and temperature.

Hospital Stays

During your visit you will be restricted to the CRC. Although quiet leisure activities can be provided, confinement to the CRC may be boring. You may move around your assigned hospital room at will or use the facilities as needed, unless the test being performed requires bed-rest. Some people may find it hard to be confined to a hospital bed for the duration of the tests.

Psychological Stress

Some of the questions we will ask you as part of this study may make you feel uncomfortable. These would be questions about your normal food and alcohol consumption, and your physical activity. You may refuse to answer any of the questions, take a break, or stop your participation in this study at any time. You will get a chance to see the test during the screening procedures.

Alcohol Consumption

You will be asked to consume vodka during the metabolic study, every hour from noon to 5 PM. The first dose at noon is based on your body weight and is designed to bring you to a blood alcohol level of 0.10 within an hour. This amount is roughly equivalent to 5 drinks consumed in 1 hr for a 200-lb person. Your breath alcohol level will be measured every 30 minutes using a breathalyzer and if any measurement is above 0.15, your alcohol intake will be slowed until BrAC drops to 0.10. The effects of this dosage can vary among individuals and their tolerance, but short-term effects include euphoria, impaired judgment, lethargy, sedation, delayed reactions, balance difficulty, impaired fine muscle coordination, impaired vision, confusion, inability to feel pain (analgesia), dizziness, vomiting and the increased risk of falling.. To avoid excessive side effects and for your own safety, breath alcohol concentration (BrAC) will be assessed every 30 minutes using a breathalyzer,

Hangover

Due to the amount of alcohol you will be asked to consume, there is a risk of having a hangover on the following day. The typical symptoms are dizziness, dry mouth, headache, gut complaints, nausea/vomiting and feeling of discomfort.

Risks of Radiation - DEXA

As part of this study you will be exposed to ionizing radiation through a DEXA x-ray performed once.

If you have participated in any other research study involving ionizing radiation exposure in the past 12 months, discuss this with the Investigator to determine if you are eligible to participate in this study.

You will be exposed to a radiation dose which is about 2% of the average radiation dose from all sources (natural background radiation, consumer appliances, radon gas, medical tests, etc.) that a person receives in the United States receives each year. **Radiation effects are cumulative. You should always inform future doctors of your participation in this study.**

Risks of Magnetic Resonance Spectroscopy (MRS)

You will lie in the MRI scanner for this test. The scanner makes a loud, banging noise during the scan, and you will be given a set of earplugs to help reduce the noise. You may experience nervousness from confinement in a tight space (claustrophobia). If you become anxious, you can stop the procedure at any time. You may experience some discomfort and fatigue from lying during scanning. There are no known effects from exposure to magnetic fields. If you have metal clips or plates in your body, you should tell the Investigator.

Oral Consumption of a Stable Isotope

For three days before your test and on the test day, you will consume small amounts of special heavy water with your meals. The isotope is a natural molecule called a “stable” isotope. Stable isotopes contain no radioactivity and have no recognized harmful effects. There is no risk associated with the ingestion of these labeled compounds.

Loss of Confidentiality

Any time information is collected there is a potential risk for loss of confidentiality. Every effort will be made to keep your information confidential; however, this cannot be guaranteed.

Other Risks

There may possibly be other side effects that are unknown at this time. If you are concerned about other, unknown side effects, please discuss this with the researchers.

How will risks be minimized or prevented? You will be screened before the study to ensure that you are an appropriate candidate for the study. Participants are selected so that the amount of alcohol does not exceed the amount they have consumed on their own in the recent past (past 6 months). Your risks will be minimized by techniques used by our staff who have been properly trained to monitor for any signs of discomfort during the visits and tests. If a problem occurs while you are being studied, the research procedures will be suspended and the study physician will advise as to the proper care. A nurse or licensed technician will perform the blood draws. The protocols that the CRC nurses follow to reduce risk of infection include: cleaning the skin thoroughly before IV placement, securing the IV with tape, covering the site with clean gauze, and protecting the site using an elastic sleeve. The site is inspected at each change in shift – with particular attention to any changes in the surrounding skin with

respect to swelling, color, and temperature. Research personnel will always be available during the study visits, as well as by telephone at other times.

PART 3. WHAT WILL MY RESPONSIBILITIES BE DURING THE STUDY?

While you are part of this study, the researchers will follow you closely to determine whether there are problems that need medical care. It is your responsibility to do the following:

- Ask questions about anything you do not understand.
- Keep your appointments.
- Follow the researchers' instructions, particularly in consuming the 3-day diet
- Let the researchers know if your telephone number or address changes.
- Report to the researchers any injuries or illnesses while you are on the study, even if you do not think they are related.

ARE THERE BENEFITS TO TAKING PART IN THE STUDY?

If you agree to take part in this study, there may or may not be direct medical benefit to you. You may expect to benefit from taking part in this research to the extent that you are contributing to medical knowledge. We hope the information learned will benefit others in the future. New information may lead to a better understanding of how excess alcohol and food can be harmful to the liver.

WHAT OTHER OPTIONS ARE THERE?

You do not have to participate in this study.

What about Confidentiality?

Information produced by this study will be stored in the investigator's file and identified by a code number only. The code key connecting your name to specific information about you will be kept in a separate, secure location. Information contained in your records may not be given to anyone unaffiliated with the study in a form that could identify you without your written consent, except as required by law. If the investigator conducting this study is not your primary or regular doctor, she must obtain your permission before contacting your regular doctor for information about your past medical history or to inform them that you are in this study.

It is possible that your medical and/or research record, including sensitive information and/or identifying information, may be inspected and/or copied by the study sponsor (and/or its agent), the Food and Drug Administration (FDA), federal or state government agencies, MU Health Sciences IRB, or hospital accrediting agencies, in the course of carrying out their duties. If your record is inspected or copied by the study sponsor (and/or its agents), or by any of these agencies, the University of Missouri will use reasonable efforts to protect your privacy and the confidentiality of your medical information.

The results of this study may be published in a medical journal or used for teaching purposes. However, your name or other identifying information will not be used in any publication or teaching materials without your specific permission.

WHAT ARE THE COSTS?

You will not be charged for any procedures that are part of this research study. You or your insurance company will be charged for continuing medical care and/or hospitalization.

WILL I BE PAID FOR PARTICIPATING IN THE STUDY?

You will not be paid for the screening part of the study. You will be paid \$100 after completion of the overnight stay. The researchers will also provide you with the labeled water ahead of the study, and on the treatment day, food and alcohol to be consumed.

WHAT IF I AM INJURED?

It is not the policy of the University of Missouri to compensate human subjects in the event the research results in injury. The University of Missouri, in fulfilling its public responsibility, has provided medical, professional and general liability insurance coverage for any injury in the event such injury is caused by the negligence of the University of Missouri, its faculty and staff. The University of Missouri also will provide, within the limitations of the laws of the State of Missouri, facilities and medical attention to subjects who suffer injuries while participating in the research projects of the University of Missouri. In the event you have suffered injury as the result of participation in this research program, you are to contact the Risk Management Officer, telephone number (573) 882-1181, at the Health Sciences Center, who can review the matter and provide further information. This statement is not to be construed as an admission of liability.

WHAT ARE MY RIGHTS AS A PARTICIPANT?

Participation in this study is voluntary. You do not have to participate in this study. Your present or future care will not be affected should you choose not to participate. If you decide to participate, you can change your mind and drop out of the study at any time without affecting your present or future care at the University of Missouri. Leaving the study will not result in any penalty or loss of benefits to which you are entitled. In addition, the investigator of this study may decide to end your participation in this study at any time after she has explained the reasons for doing so and has helped arrange for your continued care by your own doctor, if needed.

You will be informed of any significant new findings discovered during the course of this study that might influence your health, welfare, or willingness to continue participation in this study. A description of this study is available on **www.ClinicalTrials.gov**, as required by U.S. law. This Web site will not include information that can identify you. At most, the Web site will include a summary of

the results. You can search this Web site at any time with this study's identifier number: NCT02141880.

WHOM DO I CALL IF I HAVE QUESTIONS OR PROBLEMS?

If you have any questions regarding your rights as a participant in this research and/or concerns about the study, or if you feel under any pressure to enroll or to continue to participate in this study, you may contact the University of Missouri Health Sciences Institutional Review Board (which is a group of people who review the research studies to protect participants' rights) at (573) 882-3181. You may also contact the Research Participant Advocate (RPA) at (573) 884-1925 or (888) 280-5002 (toll-free). If you prefer email, you can reach the RPA at somrpa@missouri.edu. You may ask more questions about the study at any time. For questions about the study or a research-related injury, contact Dr. Elizabeth Parks at (682) 433-9012. A copy of this consent form will be given to you to keep.

SIGNATURES *

I confirm that the purpose of the research, the study procedures, the possible risks and discomforts as well as potential benefits that I may experience have been explained to me. Alternatives to my participation in the study also have been discussed. I have read this consent form and my questions have been answered. My signature below indicates my willingness to participate in this study.

Subject	Date	Time
---------	------	------

SIGNATURE OF STUDY REPRESENTATIVE

I have explained the purpose of the research, the study procedures, identifying those that are investigational, the possible risks and discomforts as well as potential benefits and have answered questions regarding the study to the best of my ability.

Study Representative*	Date	Time
-----------------------	------	------

*Study Representative is a person authorized to obtain consent. Per the policies of the University of Missouri Health Care, if the study is deemed either 'significant risk/non-treatment' or 'minimal risk,' the Study Representative may be a non-physician study investigator. For any 'significant risk/treatment' study, the Study Representative must be a physician who is either the Principal or Co-Investigator.

VITA

Majid Mufaqam Syed-Abdul was born in Hyderabad, India. He completed his pharmacy school at Deccan School of Pharmacy affiliated with Jawaharlal Nehru Technological University in Hyderabad and completed his PGY1 in clinical pharmacy at the Nirma University, Ahmedabad, India.

Being a frontline as a pharmacist, he was asked about nutrition/diet related questions which led him to pursue his Master of Science in Nutrition and Exercise Science at Southeast Missouri State University, Cape Girardeau, MO, USA. During his master's degree, he got interested in nutrition research and joined Dr. Elizabeth Parks' Lab as a PhD student in Nutritional Sciences at the University of Missouri, Columbia, MO, USA.

Majid completed his doctorate degree in December 2020 and plans to pursue post-doctoral fellowship in lipoprotein metabolism.