Isotope labeling and biochemical assessment of liver-TAG in patients with different levels of histologically-graded liver disease

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A1. Supplementary Methods

A1.1. Liver tissue processing and histological scoring

Once liver tissue was obtained, the sample was immediately transferred to the research lab on ice and was weighed instantly in a 0.9% sterile sodium chloride solution (#306546, BD PosiFlush™, Franklin Lakes, NJ). Approximately 50 mg of tissue was fixed in 10% neutral buffer formalin and was stored at 5°C before sending the tissue to a histopathologist for the examination. The remaining tissue was frozen and stored at -80°C freezer for other measurements. Liver histological scoring was performed by an experienced hepatopathologist (Alberto Diaz-Arias, MD). Hematoxylin-eosin and Masson's trichrome staining was performed according to the Brunt scoring scale for NAS and fibrosis score [35, 36].

A1.2. Western blotting

For western blotting, liver tissue was washed with ice-cold PBS and lysed with a buffer solution, processed and probed as described previously 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 ChemiDocTM 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 [37, 38]. 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 **supplementary table 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−ΔΔCT method [37, 39]. All analyses were performed without the knowledge of the histologic score.

A1.3. Methodological details for measurement of liver-TAG and -CE content, fatty acid (FA) composition, and DNL

For liver-TAG, approximately 30 mg of tissue was extracted using the Folch method [1], homogenized for two minutes, and exposed to agitation overnight. One ml of magnesium chloride (4 mM) was added, vortexed, and the solution centrifuged for one hour at 1000g at 4^{o}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 TAG was measured using a commercially available kit (#G7793, Sigma, St. Louis, MO) and the final values are reported in mg/g of tissue wet weight [2]. For liver-TAG and -CE fatty acid *composition,* 100mg of tissue was homogenized using Bio-Gen homogenizer (#PRO200, PRO Scientific Inc., Oxford, CT) in six ml Folch solution (chloroform and methanol in 2:1 ratio) containing the internal standard for CE-17:0 (#CH-816-S5-C, Nu-Chek Prep, Inc., Elysian, MN) in a proportion as expected in normal and disease liver [3]. Total lipids were extracted using the method described previously [1]. The liver-TAG and -CE was separated via thin-layer chromatography (TLC) and fatty acid methyl esters (FAME) prepared as described previously [4]. The fatty acid compositions of liver-TAG and -CE and VLDL-TAG and -CE were quantitated by gas chromatography with flame ionization detection, using a 7890B gas chromatography (Agilent Technologies, Palo Alto, CA) using a DB-23 column (60m length, inner diameter 0.250mm, 0.15µm 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 against a mixed fatty acid standard. For CE, the concentration of CE-17:0 was used to calculate the concentration of each

fatty acid, and the known molecular weight of that fatty acid in CE was used to calculate the total liver CE concentration, reported as mg/g of tissue wet weight.

A1.4. DNL measurements

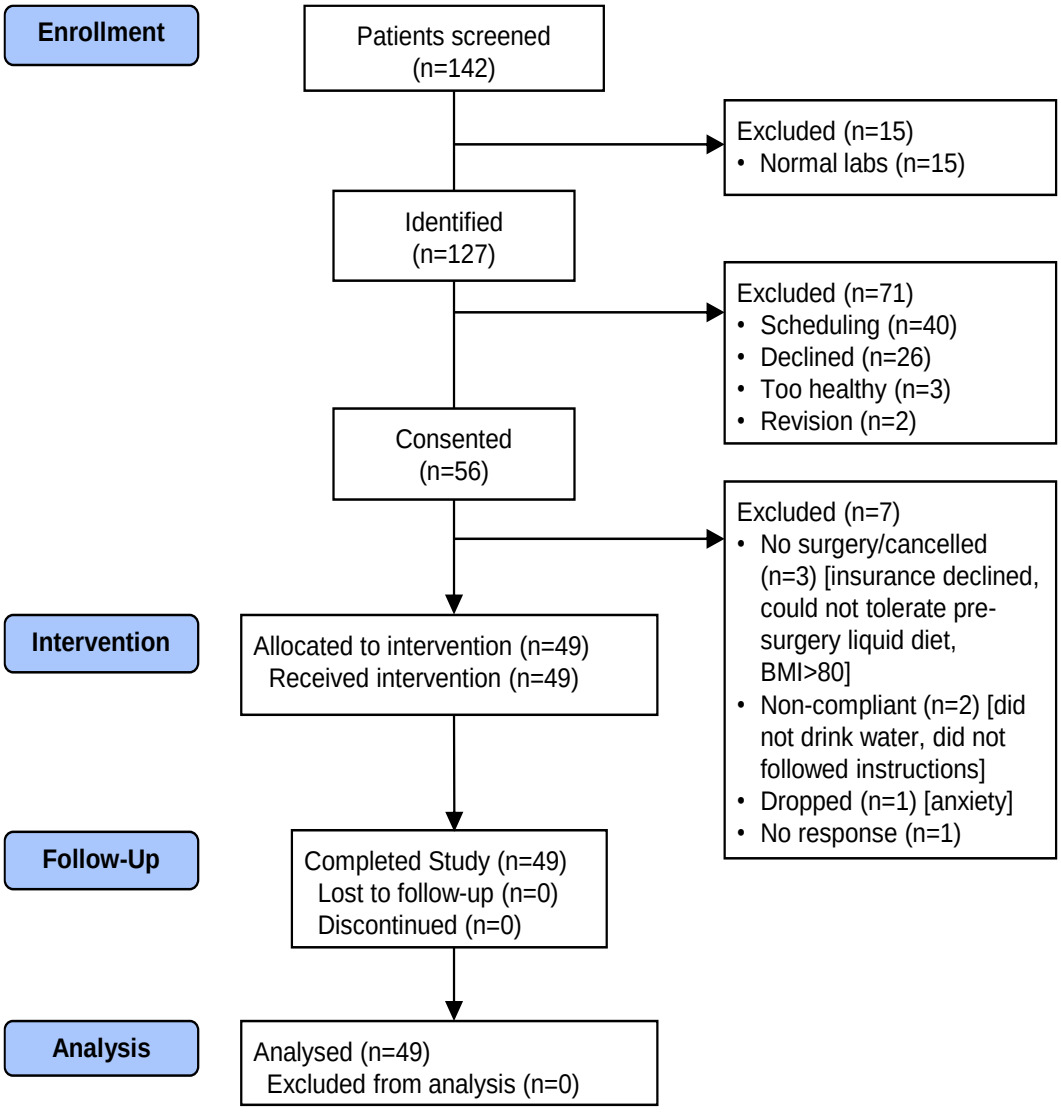
To label lipogenesis in vivo, d_2O was purchased from Cambridge Isotope Laboratory, Inc. (70% concentration, purity ≥99.5%, Catalog# DLM-4-70-0, Andover, MA) and subjects consumed the label by mouth according to the plan shown in **fig. S2**. Final d_2O enrichments in plasma were measured by cavity ringdown 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). The average body d_2O enrichments were 0.52±0.14% (mean±SD). Very low-density lipoprotein (VLDL) particles were isolated at 40,000 rpm for 20h in a Beckman 50.3 Ti rotor via ultracentrifugation (1.3×10^8g) and the top two ml were collected using tube slicing [5]. VLDL lipids were isolated and processed by TLC as described above for liver lipids [5]. All FAME from liver-TAG and -CE, and VLDL-TAG and -CE were analyzed for isotopic enrichment on an Agilent 6890N gas chromatography coupled to a 5975 mass spectrophotometer (Agilent Technologies, Palo Alto, CA). FAMEs 14:0, 16:0, and 18:0 made in the DNL pathway was quantitated using mass isotopomer distribution analysis [6, 7]. The fractional DNL (in units of %) reflects intrahepatic assembly of lipid and is a read-out of whether one lipid source is preferred over another (nonDNL derived from the diet or adipose FFA) for intrahepatic TAG synthesis. By contrast, the total liver fatty acid synthesis is presented in units of mg/g liver and referred to as absolute DNL (absDNL). AbsDNL is calculated by multiplying the percentage DNL (14:0, 16:0, and 18:0) by total lipid concentration (e.g., percent DNL in 16:0 fatty acid from liver-TAG is multiplied by total liver-TAG 16:0 concentration) [8]. In VLDL, the absDNL has units of mg/dL and represents the total quantity of 14:0, 16:0, and 18:0 fatty acids made de novo that are carried in the VLDL particles in plasma.

A2. Supplementary References

1. J Folch, M Lees, GH Sloane Stanley. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 1957; 226(1):497-509. PMID 13428781
2. RS Rector, JP Thyfault, RT Morris, MJ Laye, SJ Borengasser, FW Booth, JA Ibdah. Daily exercise increases hepatic fatty acid oxidation and prevents steatosis in Otsuka Long-Evans Tokushima Fatty rats. Am J Physiol Gastrointest Liver Physiol 2008; 294(3):G619-626. PMID 18174272
3. P Puri, RA Baillie, MM Wiest, F Mirshahi, J Choudhury, O Cheung, C Sargeant, MJ Contos, AJ Sanyal. A lipidomic analysis of nonalcoholic fatty liver disease. Hepatology 2007; 46(4):1081-1090. PMID 17654743
4. KL Donnelly, CI Smith, SJ Schwarzenberg, J Jessurun, MD Boldt, EJ Parks. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. J Clin Invest 2005; 115(5):1343-1351. PMID 15864352
5. BR Barrows, EJ Parks. Contributions of different fatty acid sources to very low-density lipoprotein-triacylglycerol in the fasted and fed states. J Clin Endocrinol Metab 2006; 91(4):1446-1452. PMID 16449340
6. MK Hellerstein, RA Neese. Mass isotopomer distribution analysis at eight years: theoretical, analytic, and experimental considerations. Am J Physiol 1999; 276(6):E1146-1170. PMID 10362629
7. MK Hellerstein, M Christiansen, S Kaempfer, C Kletke, K Wu, JS Reid, K Mulligan, NS Hellerstein, CH Shackleton. Measurement of de novo hepatic lipogenesis in humans using stable isotopes. J Clin Invest 1991; 87(5):1841-1852. PMID 2022750
8. EJ Parks, LE Skokan, MT Timlin, CS Dingfelder. Dietary sugars stimulate fatty acid synthesis in adults. J Nutr 2008; 138(6):1039-1046. PMID 18492831

A3. Supplementary Figures

Figure S1. Flow diagram describing patient recruitment and enrollment



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, or were undergoing a correction/revision surgery. A total of 56 patients signed the consent form but three patients' surgeries were canceled, two patients did not consume deuterated water (d_2O), one patient dropped out, and one patient did not respond after consenting. A total of 49 patients completed the study and their data were used for analyses.

Figure S2. Study design

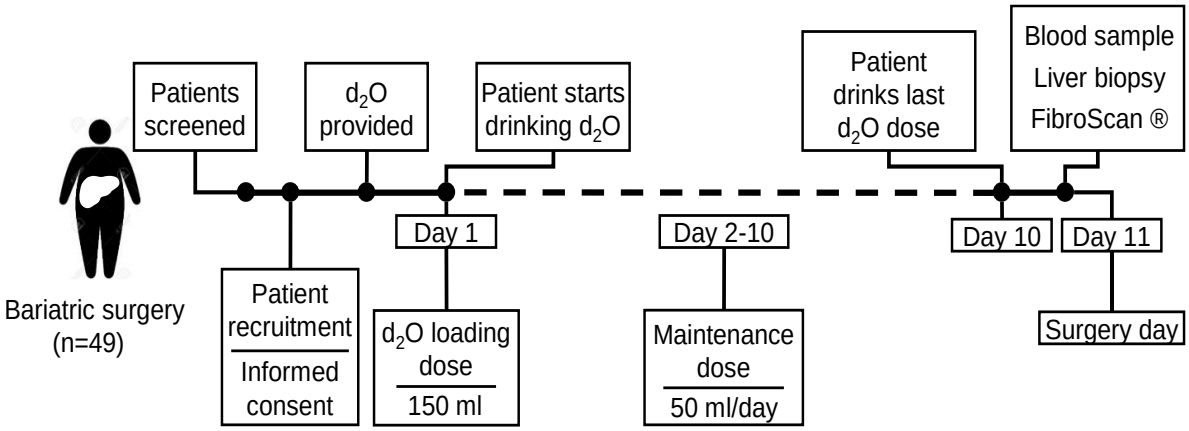
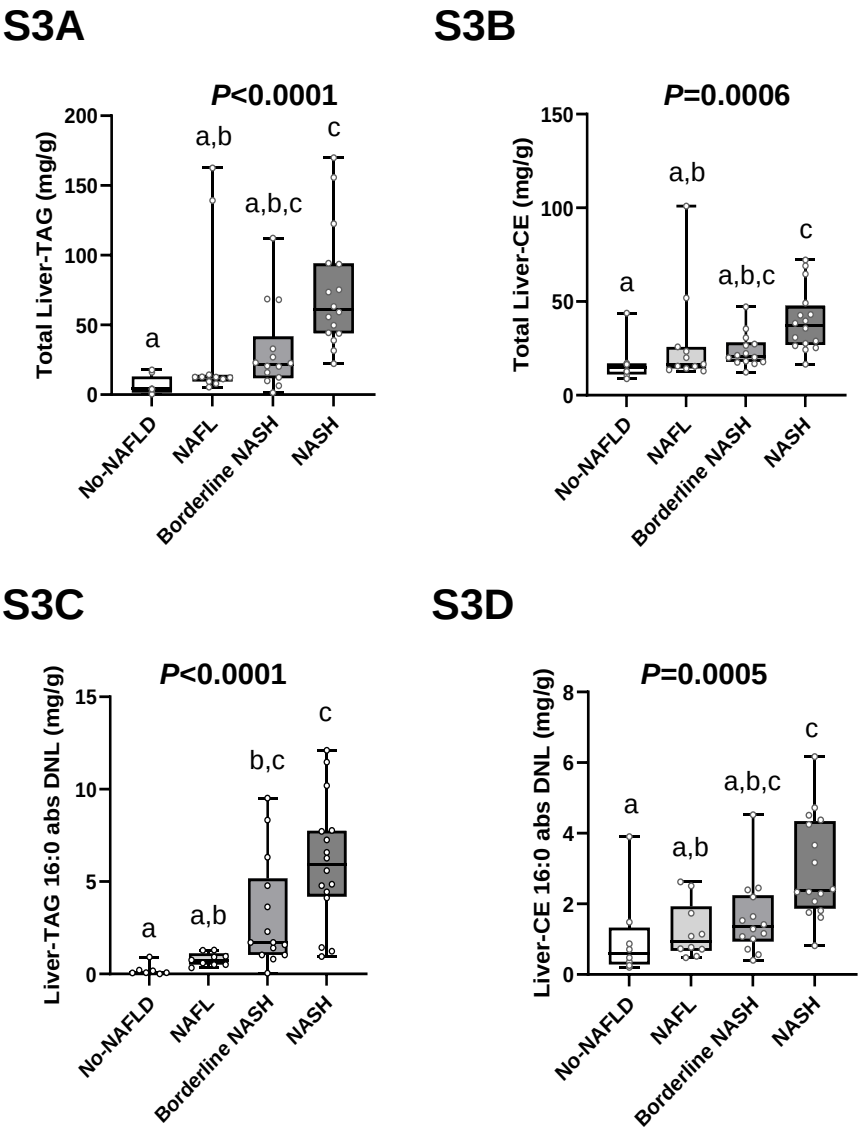


Figure S3. Relationship between liver-TAG and CE, and de novo lipogenesis (DNL) in liver TAG and CE



Data are reported as median with confidence interval. Total n=49, No-NAFLD n=8, NAFL n=11, Borderline NASH n=14, NASH n=16, unless otherwise noted.
The Kruskal-Wallis ANOVA was performed between the groups and the *P*-value is presented above each bar graph. If significant, Dunn's multiple comparisons test was performed to test the significance of each group. All liver lipid was presented in units of mg lipid per g liver tissue wet weight. For comparisons that were significant, superscript letters that are not shared represent values that are significantly different from one another.
A) Triacylglycerol (TAG) content across the liver groups.
D) Cholesterol ester (CE) content across the liver groups.
C) Absolute DNL, calculated by multiplying the percent of newly-made 16:0 by the total liver-TAG 16:0 content. Total n=46, No-NAFLD n=7, NAFL n=9, Borderline NASH n=14, NASH n=16
F) Absolute DNL in liver-CE 16:0.

A4. Supplementary Table

Table S1. Primer sequences for SYBR Green quantitative real-time PCR

Primer	Sequence 5' ~ 3'	Name/synonym
ACC1	F: ACA TTA AGA TGG CAG ATC R: CTT GTA CTG GGA TCT TT	Acetyl-coenzyme-A carboxylase-1
CD36	F: AGC TTT CCA ATG ATT AGA CG R: GTT TCT ACA AGC TCT GGT TC	Fatty acid translocase
DGAT1	F: ATC TTC TTC TAC TGG CTC TTC R: CAG AAG TAG GTG ACA GAC TC	Diacylglycerol o-acyltransferase-1
DGAT2	F: GAG ACT ACT TTC CCA TCC AG R: GAA CTT CTT GCT CAC TTC TG	Diacylglycerol o-acyltransferase-2
FASN	F: CAA TAC AGA TGG CTT CAA R: GAT GTA TTC AAA TGA CTC	Fatty acid synthase
GAPDH	F: AAC AGC CTC AAG ATC AGC AA R: CAG TCT GGG TGG CAG TGA T	Glyceraldehyde 3-phosphate dehydrogenase
HMGCR	F: ACT TCG TGT TCA TGA CTT TC R: GAC ATA ATC ATC TTG ACC CTC	3-Hydroxy-3-methylglutaryl-coenzyme-A reductase
NCEH1	F: CAG TTT ACT CAA GAT GCC AG R: CCT GCA CAA AGT CAT AGT TG	Neutral cholesterol ester hydrolase
SCD	F: CAG AGG AGG TAC TAC AAA CC R: ATA AGG ACG ATA TCC GAA GAG	Stearoyl-coenzyme A desaturase
SOAT2	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)