Supplementary Materials

Postprandial plasma lipidomics reveal specific alteration of hepatic-derived diacylglycerols in non-alcoholic fatty liver

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Supplemental Methods

Clinical trial design

Adult (age >=18) subjects were eligible for inclusion if they had biopsy-proven NAFLD within 2 years of enrollment, or if they had likely NAFLD, defined as at least 2 of the following criteria: elevated ALT, fatty liver by imaging, and metabolic syndrome or diabetes. Fatty liver was confirmed by imaging in all subjects. Subjects were excluded if they had other concurrent liver diseases, decompensated cirrhosis, excessive alcohol consumption (>30 g/d for men or >20 g/d for women or binge drinking), >10% weight change within 6 months, disorders or medications interfering with intestinal absorption, pregnancy or lactation. Diabetic patients were eligible for enrollment unless they were treated with insulin.

Adult healthy controls were eligible for enrollment if they had normal BMI, normal transaminases (alanine aminotransferase (ALT) ≤ 31 U/L for men or ≤ 19 U/L for women, and aspartate aminotransferase (AST) ≤ 30 U/L), fasting glucose <95 mg/dL, no liver fat by ultrasound, no history of liver disease, and no use of chronic medications with the exception of oral contraceptives. A third cohort was planned with subjects with metabolic syndrome and no evidence of NAFLD. However, all candidates with metabolic syndrome who were screened for this cohort were found to have NAFLD by imaging and/or elevated liver enzymes. In its place, we obtained samples from a similar study of obese subjects without evidence of NAFLD (see below).

Enrolled subjects were admitted to the NIH Clinical Center a day before testing and received a standardized dinner at 18:00. After an overnight fast, on the day of testing, subjects entered a whole-room indirect calorimeter (metabolic chamber)[1], where they stayed for 24 hours. At 08:00, subjects were given a single dose of a liquid meal (Ensure Plus, Abbott) containing 30% of their total estimated daily energy needs, calculated using the Mifflin St. Jeor equation[2] with an activity factor of 1.4. The meal composition was 350 kcal per 8 oz. (237ml), with 57% of calories from carbohydrates, 28% from fat and 15% from protein and subjects were instructed to consume it within 15 minutes. Subjects remained sedentary for 4 hours after the meal.

Blood samples were collected prior to the test meal, and at 0.5, 1, 2 and 4 hours after it. Samples were placed on ice, separated into plasma and serum, aliquoted and stored at -80C. Following the 4-hour blood draws, subjects remained in the metabolic chamber and received standardized lunch and dinner, again at 18:00. After an overnight fast, a final blood sample was drawn the following morning, 24 hours after the test meal and prior to breakfast, and was processed in a similar manner.

Baseline energy expenditure was measured in the metabolic chamber and defined as the average values over a period of 30 minutes at rest after chamber equalization. Baseline respiratory quotient was measured as the ratio of CO_2 produced to O_2 consumed over the same period of time and post-prandial respiratory quotient was the average ratio over the 4 hours following the meal. Body composition was assessed by dual energy x-ray absorptiometry scanner (iDXA scanner with Encore 11.10 software; GE Healthcare, Madison, WI, USA).

Non-NAFLD Obese Subjects

Obese non-NAFLD subjects were selected from an ongoing cross-sectional study at the NIH Clinical Center (clinicaltrials.gov NCT00428987). The study was approved by the NIDDK/NIAMS Institutional Review Board and all subjects provided written informed consent. Study procedures and inclusion criteria were as previously described[3]. Subjects underwent a mixed meal test after a 12-hour overnight fast with a liquid meal containing at a fixed dose of 360 kcal (50% from carbohydrates, 35% from fat and 15% from protein, Boost Plus). Blood samples were drawn at baseline and 3 hours after the meal and plasma was separated, aliquoted, and stored as described above. For the purpose of this study, subjects were selected if they had BMI >=30 and liver fat content <=5% by ^{1}H-magnetic resonance spectroscopy.

Animal Studies

Eight-week-old C57Bl/6N male mice were randomized into two groups and given the Gubra Amylin NASH (GAN) diet[4] (D09100310) or a matching control diet (D17112301R,

Research Diets, Inc.) for 8, 16 or 26 weeks. Mice were housed under standard 12-hour light cycle with access to food and water *ad libitum*. Body weight and food consumption were recorded weekly. At the endpoint, lean and fat body mass composition was determined by Echo MRI (EchoMRI-2010) and all mice were fasted from 12:00-18:00. At 18:00, mice fed control or GAN-diet for 26 weeks were given, by gavage, a single dose of a liquid meal (Ensure Plus) at 25 µL/g lean body mass and euthanized 0.5, 1, 2, or 4 hours later. Mice euthanized at 0 hours were given 25 µL/g water by gavage immediately prior to euthanasia. In additional experiments, mice fed control or GAN-diet for 8- and 16-weeks were fasted and given a liquid meal as described, then euthanized at 1 hour.

To determine the contribution of lipids from the liquid meal to postprandial plasma DAGs, mice fed control or GAN-diet for 26 weeks were fasted and given a liquid meal by gavage as described, with the addition of ^{12}C- or ^{13}C-triolein (1,1,1-^{13}C3, Cat# CLM-163, Cambridge Isotope Laboratories Inc., Tewksbury MA) at a 1:1 ratio (27 mM) with Ensure Plus oleic acid content (81 mM) and euthanized at 1 hour.

To characterize a “second meal effect” with contribution of lipids from a previous meal to postprandial plasma DAGs, mice were given ^{13}C-triolein in Ensure Plus at 12:00, fasted from 12:00-18:00 then provided with a second liquid meal of Ensure Plus. Blood was obtained immediately prior to and 1 hour following liquid meal administration.

The contribution of peripheral lipases to the generation of postprandial plasma DAGs was determined in mice fed control or GAN-diet for 26 weeks by intraperitoneally injecting fasted mice with saline or the lipolysis inhibitor tyloxapol (500 mg/kg) 30 minutes prior to a liquid meal gavage. Blood was obtained immediately prior to and 1 hour following the meal.

Sample processing and lipidomic analysis

Plasma and liver sample processing

Plasma lipids were extracted using the Folch method, as previously described[5]. Briefly, five volumes of 2:1 chloroform:methanol containing 0.5 µM C17 ceramide (d18:1/17:0) (Cat# 860517, Avanti Polar Lipids, USA) as internal standard were added to one volume of plasma. Samples were vortexed and centrifuged at 13,000 g for 10 minutes. Liver lipids were extracted using a modified Bligh & Dyer method. A ~20 mg piece of liver tissue was homogenized in 14 µl/mg tissue of 3:4 water:methanol using a Precellys tissue homogenizer (6500 RPM for 30s x 2; Bertin Technologies, France). Chloroform containing 0.5 µM C17 Ceramide (d18:1/17:0) was added to each sample at a ratio of 16 µl/mg tissue. The organic layer from plasma and tissue samples was transferred to a glass tube and dried under a gentle stream of nitrogen. Plasma lipids were reconstituted in one volume of 1:1 chloroform:methanol followed by dilution in four volumes of isopropanol/acetonitrile/water (50/25/25). Liver lipids were reconstituted in isopropanol/methanol/chloroform (4/2/1). A 5 µL or 1 µL sample of extracted plasma or liver lipids, respectively, was subjected to ultra-performance liquid chromatography coupled to quadrupole time of flight mass spectrometry (UPLC-QTOFMS) or tandem mass spectrometry (UPLC-MS/MS) as described below.

Untargeted lipidomics and stable isotope enrichment UPLC-QTOFMS analysis

Samples were randomized and injected into a Waters I-Class ultra-performance liquid chromatography (UPLC) system. A pooled sample was injected after every ten samples as a quality control. Lipids were separated using an ACQUITY UPLC CSH C18 1.7 µm column (2.1 x 100 mm) maintained at 55°C. The mobile phase buffer A consisted of acetonitrile/water (60:40) with 10 mM ammonium formate and 0.1% formic acid and mobile phase buffer B consisted of isopropanol/acetonitrile (90:10) with 10 mM ammonium formate and 0.1% formic acid. The UPLC flow rate was 400 µL/min and gradient conditions were as follows: 0–2 mins, 40–43% B; 2.1–12 mins 50–54% B; 12.1–18 mins 70–99% B; 18.1 mins 40% B. UPLC-QTOFMS analysis was performed in a Waters SYNAPT G2-Si in positive and negative electrospray ionization modes. The following instrument conditions were used: 2.8 kV capillary voltage, 150°C source temperature, 40 V sampling cone, and a desolvation gas flow rate of 950 l/hour at 500°C. The data was acquired in centroid mode with a 0.1 s scan time in the range of

100–1200 m/z by data-independent acquisition using an MS^E method in Masslynx 4.2. The low collision energy was set to 4 V and high collision energy was ramped from 10 to 45 V. Leucine-enkephalin was used for lock mass correction.

Untargeted lipidomics data processing and lipid identification

For untargeted lipidomics analysis, peak picking, deconvolution, adduct annotation and retention time alignment was performed on positive and negative ionization mode raw data files using Progenesis QI software. Peak areas were normalized to internal standard and features with a relative standard deviation greater than 30% across pooled injections were considered unreliable and excluded from further analysis. Features that ionize in both positive and negative mode were filtered by choosing the more sensitive ion and combined for statistical analysis and lipid identification.

Human plasma samples from 6 control and 12 NAFLD subjects were randomly chosen from the samples and processed by MS-DIAL (4.00) to generate fragmentation spectra for individual features in positive and negative ionization modes. Lipid and lipid fragment isotopes were differentiated from true lipid fragments by calculating the maximum number of carbons for a given m/z and determining if the ion intensities matching the isotope pattern were above the threshold of natural carbon isotope abundance. Ion intensities at or below predicted natural carbon isotope abundance were removed. Acylcarnitine, cholesterol ester, ceramide, diacylglycerol, fatty acid, glycerophosphocholine, glycerophosphoethanolamine, glycerophosphoserine, glycerophosphoglycerol, glycerophosphate, glycerophosphoinositol, lysophosphatidylcholine, sphingomyelin, and triacylglycerol lipid species from LipidBlast (version 10,[6]) were extracted from the data as follows: 1) The precursor lipid m/z for each lipid isomer was identified within a 5 ppm window in the feature list, 2) adducts and lipid fragment characteristic shared within each lipid class and specific to individual isomers were matched within the fragmentation spectra for each feature hit, 3) only features that matched all precursor, adduct and fragment criteria were considered identified. For lipids that ionize in both positive and negative polarities, a corresponding

isomer match was necessary within a retention time window, 4) identified features were joined with the Progenesis QI feature list by m/z and retention time.

Targeted plasma and liver DAG analysis

For targeted plasma and liver DAG analysis, lipids were separated using the same column and mobile phase as described above with the following UPLC conditions: 0–0.5 mins, 75% B; 0.5–5 mins 75–90% B; 5.01–6 mins 99% B; 6.01–8 mins 75% B. UPLC-MS/MS analysis was performed on a Waters Xevo TQ-S using multiple reaction monitoring. The cationic dehydrated monoacylglycerol product ions from the ammonium adduct of each DAG were targeted to quantify individual isomers (Table S4). DAG (36:3)(18:1-18:2) and DAG (36:3)(18:1-18:2) were synthesized by Chem-Master International, Inc (Hauppage NY) and DAG (36:4)(18:2-18:2) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Total DAG abundance was measured by summing the abundance of all measured DAG species from the targeted analysis.

Isolation of postprandial VLDL

Chylomicrons and VLDL, the triacylglycerol rich fraction (TRF) were isolated from human plasma as follows. 200 µL NaCl (0.9% w/v, G-Biosciences, USA) were under layered with 800 µl plasma in a Beckman Polypropylene Microfuge Tube (1.5 ml). Samples were centrifuged at 38,000 rpm (88712 x g) in a Beckman Optima TLX Ultracentrifuge (TLA45 rotor) for 24h at 4˚C. 150 µL of the top layer were transferred to a fresh tube. VLDL isolation was initially verified by agarose gel electrophoresis gels (Sebia Hydragel 15 lipoprotein(e) gels; Sebia Inc., USA) alongside with lipoproteins isolated by sequential density ultracentrifugation, followed by staining with Sudan Black (Fig. S4). To isolate VLDL, 50 µL of Dynabeads (Protein G, Thermo Fisher) were incubated overnight with 50 µL Anti-ApoB100 Antibody (R&D Systems, USA Cat# AF3260, RRID:AB_663782) and 200 µL 0.1% Tween in PBS on a rotating shaker at 4 ˚C. After the incubation, the beads were gently washed 3 times with 100 µl 0.1% Tween and the supernatant was discarded. Beads were then incubated with 50 µL of the TRF fraction and 50 µL 0.1% Tween for 8h, rotating at 4°C. The beads were then coupled to

the ApoB100 containing VLDL particle. The procedure was repeated two more times and all the beads were pooled for further analysis.

Targeted analysis of DAG 36:3, DAG 36:4 and DAG 36:5 from Triacylglycerol Rich Fraction (TRF) and VLDL by UPLC-MS/MS

Quantification of DAGs was achieved by ultra-performance liquid chromatography - tandem mass spectrometry (UPLC-MS/MS) utilizing a Thermo Scientific Vanquish UPLC and a Thermo Scientific Altis triple quadrupole mass spectrometer with heated electrospray ionization (HESI-II, Thermo Scientific) in positive ion mode (3500V).

DAG standard stock solutions for DAG(36:3)(18:1-18:2), DAG(36:4)(18:2-18:2), and DAG(36:5)(18:2-18:3) were prepared in methanol at concentrations from 1.0 to 5,000nM. All standard stock solutions were kept at 4°C. The internal standard solution was prepared in methanol containing 30 ng/mL of d7-C4 (C4 is 7α-hydroxy-4-cholesten-3-one). Calibration standards and VLDL samples were prepared by adding 50 µL to 200 µL methanol internal solution into a 1.7 mL centrifuge tube, vortexed for 30 minutes, and then centrifuged at 4°C, 14,000 rpm for 15 minutes. The supernatant (~200 µL) was transferred to an LC-MS vial. Injection volume was 2 µL. High-performance liquid chromatography (HPLC) grade solvents were purchased from Sigma-Aldrich. Chromatographic conditions were as follows: Solvent A: H2O with 5mM ammonium acetate, Solvent B: methanol with 5mM ammonium acetate. The flow rate was 400 µL/min and the gradient were 78% B at 0 min for 1.5 min then increasing to 93% B at 2 min, increasing at 99% B at 10 min until 13 min and returning to 78% B at 14 min. The total running time was 15 min. A Waters Acquity UPLC BEH C18, 2.1 x 100 mm, 1.7 µm column was used. The column was maintained at 50°C and the samples were maintained in the autosampler at 4°C.

Quantitation of DAGs were based on the following m/z transitions. DAG (36:3)(18:1-18:2) 636.5→339.25 and 601.57, DAG (36:4)(18:1-18:2) 634.45→599.54 and 617.54, DAG (36:5)(18:2-18:3) 632:55→597.57 and 615.54, internal standard d7-C4 408.5→177 and

390. Calibration curve had a minimum $R^2 \geq 0.99$ with 1/x weighting. Calibration and repeat measurement of standards exceeded FDA LC-MS guidelines for linearity and quantitation.

Western Blot

Bead bound particles were eluted by boiling in SDS Sample Buffer (Biorad, USA). Samples were separated on an SDS Gel under reducing conditions and transferred to a PVDF membrane. Membranes are blocked for 1 h in 5% Milk (in TBST) and incubated with Anti-ApoB overnight (1:200, 5% Milk, R&D Systems Cat# AF3556, RRID:AB_573025). Anti-ApoB recognizes both ApoB48 and ApoB100. Membranes are incubated in secondary antibody for 1 h (1:20.000, anti-goat, 5% Milk) and bands are detected using ECL (Thermo Fisher Scientific) and band intensity is recorded using the FluorChem System (Protein Simple).

Histological Analysis

Formalin-fixed liver tissue was processed and paraffin embedded. Sections were stained with hematoxylin and eosin as well as sirius red. OCT-embedded tissues were sectioned and stained with oil red O (ORO). Histological sectioning and staining were performed by HistoServ, Inc (Germantown, MD, USA). Liver histology was assessed by a blinded observer.

Plasma Aminotransferase Assay

Mouse plasma AST and ALT were determined using AST and ALT Assay Kits, according to manufacturer's protocols (Catachem, Bridgeport, CT).

Serum Insulin Levels

Serum insulin levels in human samples were measured by ELISA (R&D).

Genotyping

Genomic DNA was extracted from whole blood using the Qiagen FlexiGene DNA Kit. SNP genotyping was determined by allelic discrimination using TaqMan® Pre-Designed

SNP Genotyping Assays (Thermo Fisher Scientific, Cat# PN4351379), following the manufacturer's protocol. Briefly, 10 ng of genomic purified DNA were mixed with the corresponding TaqMan® Pre-Designed SNP Genotyping Assay (C_7241_10 for PNPLA3 rs738409 and C_89463510_10 for TM6SF2 rs58542926) and Taqman Genotyping Master mix (Thermo Fisher Scientific, Cat# 4371353) to a final volume of 5 ul. Amplifications were carried out in a 384-well plate on ViiA 7 Real-Time PCR System (Applied Biosystems).

Sample Size Calculation

No published data were available at the time of study design to allow for meaningful sample size calculation. We aimed to recruit up to 40 subjects with NAFLD and up to 20 healthy controls. An interim analysis was planned after the first 10 subjects were recruited from each arm, to generate effect size estimates and reevaluate sample size, without hypothesis testing. The prespecified interim analysis identified differential kinetics of DAGs and values obtained for DAG (36:5)(18:2-18:3) were utilized for power calculations using GLIMMPSE[7]. Power calculation was based on repeated-measures 2-way ANOVA. We calculated that if we completed enrollment of 40 NAFLD subjects and capped enrollment of healthy controls at the 10 subjects already enrolled, the power to detect a significant group*time interaction will be 97.4% at α=0.05, 89.6% at α=0.01, and 0.67 at α=0.001, respectively. We therefore decided to stop enrollment of healthy controls and complete enrollment of all pre-planned NAFLD subjects. To avoid a batch effect all samples were re-run for the final analysis.

Supplemental References

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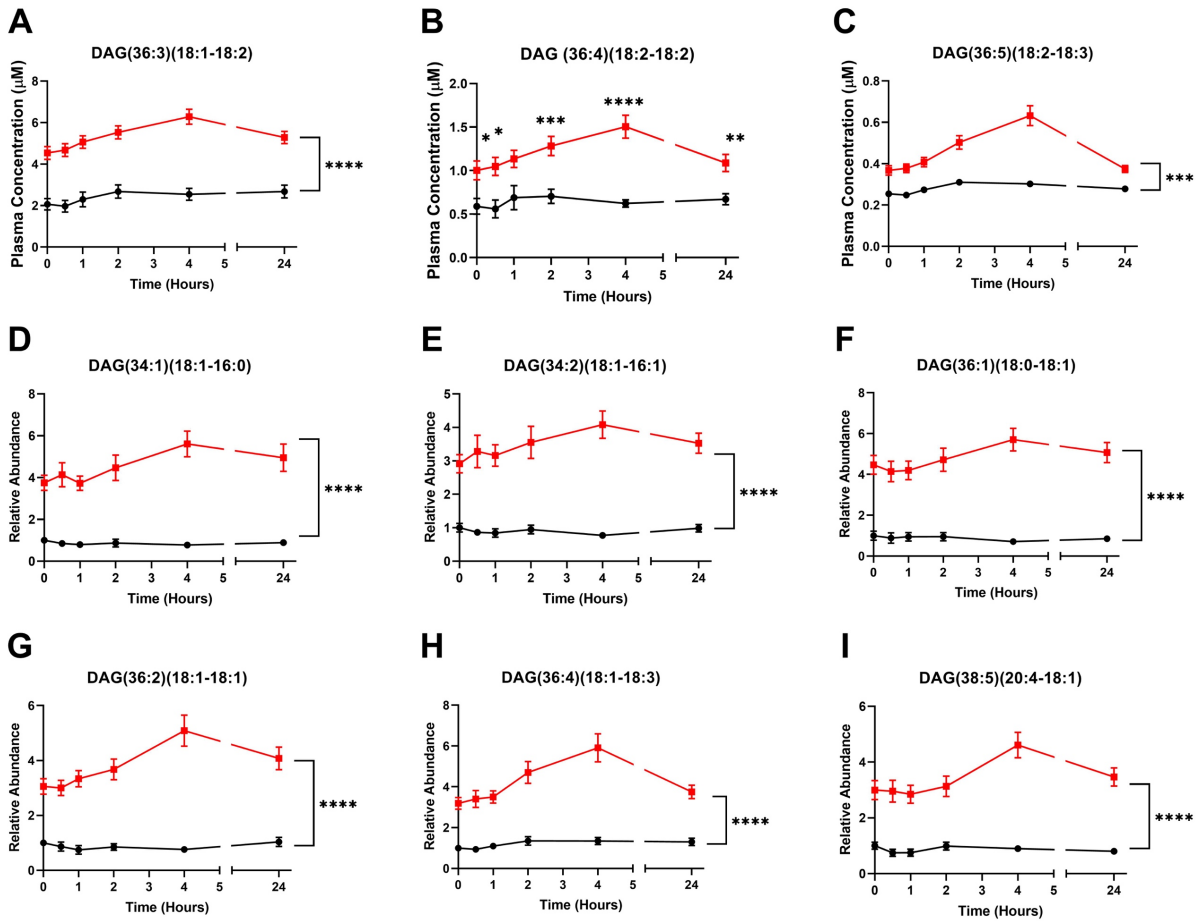
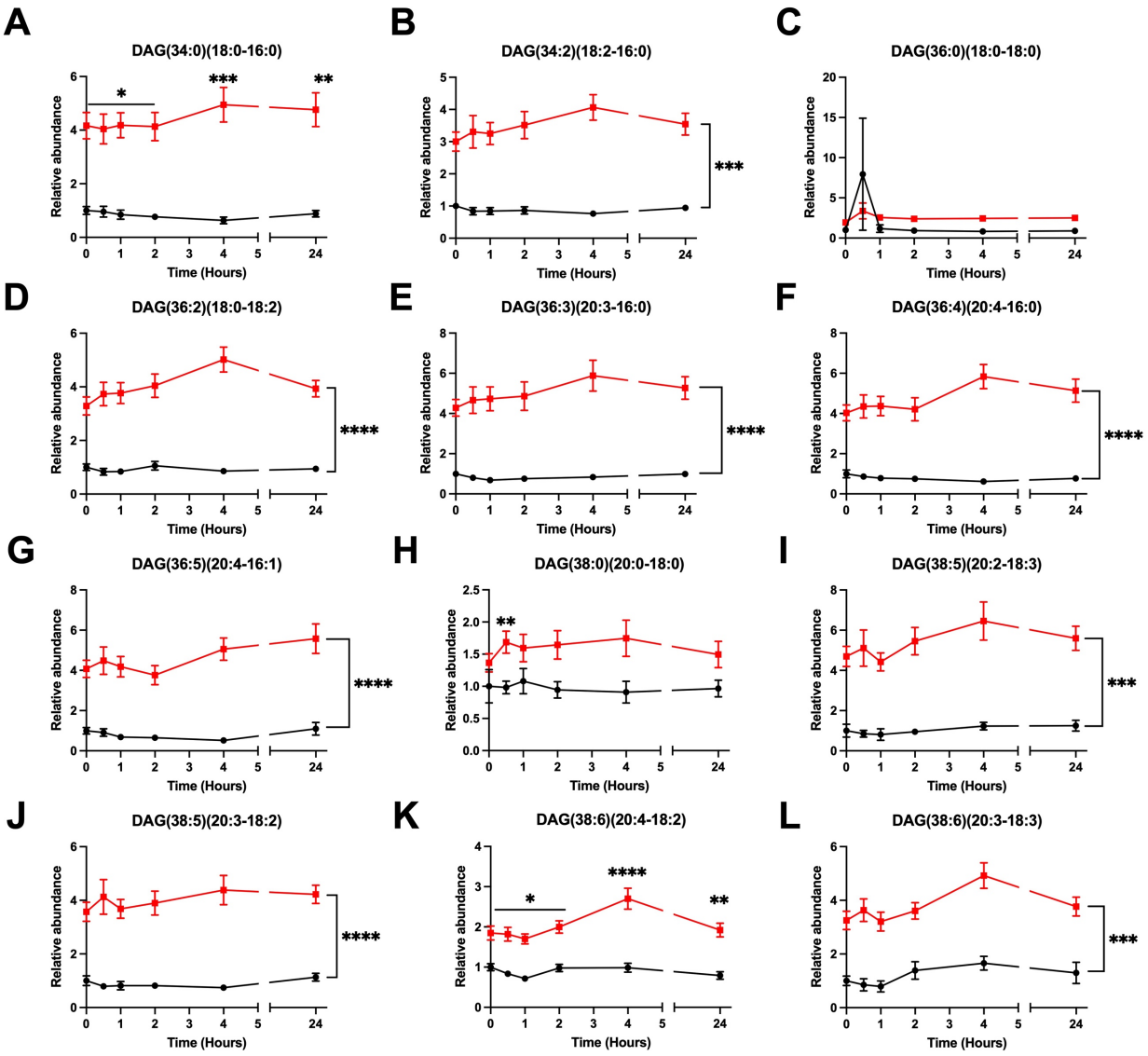
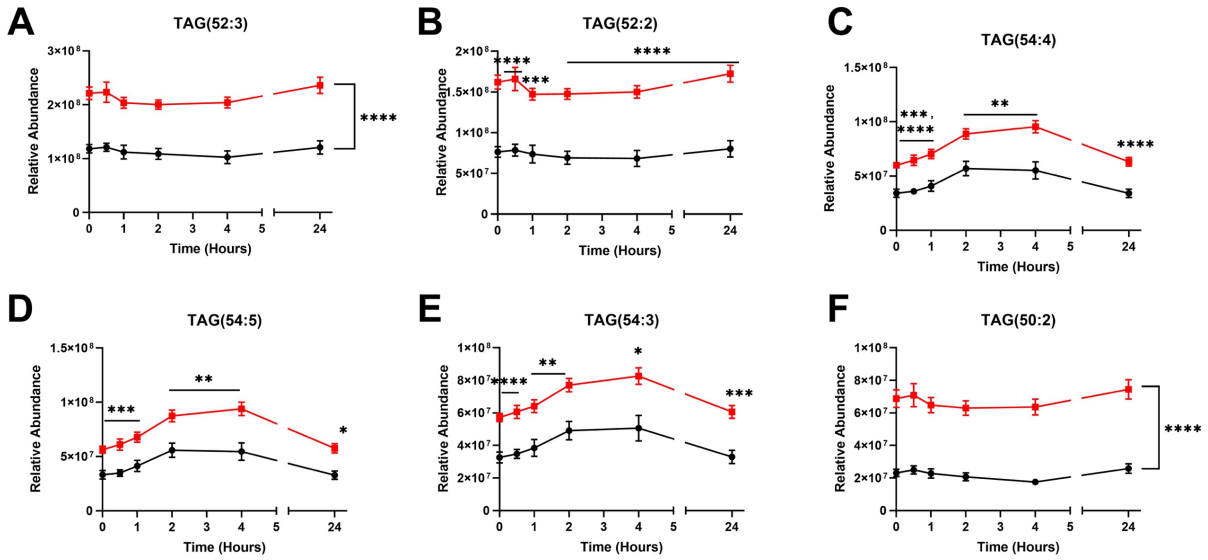
Supplemental Figures



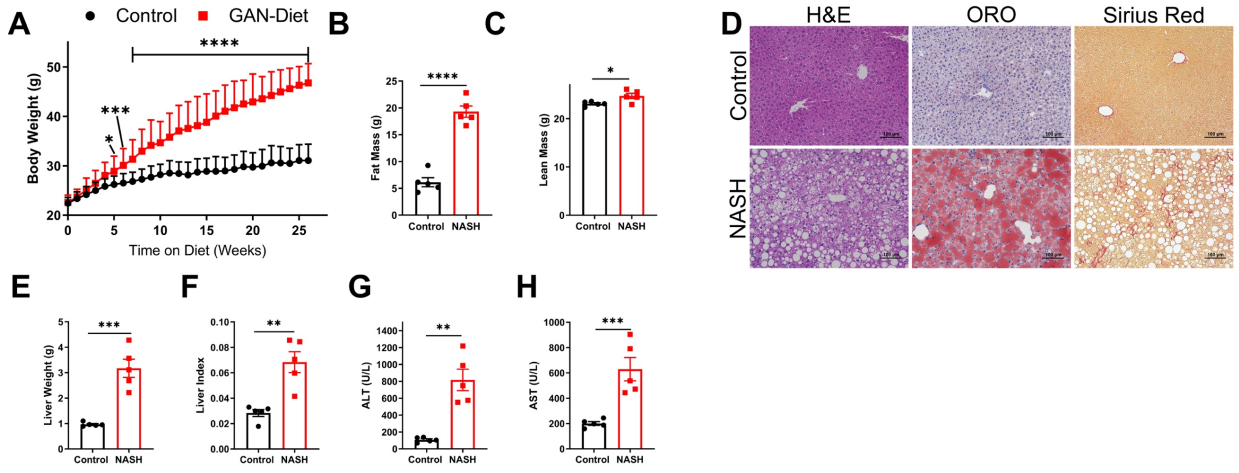
Supplemental Figure 1. Human NAFLD causes a sustained postprandial increase in plasma diacylglycerols. (**A-C**) Quantification and (**D-I**) relative abundance of significantly altered postprandial plasma diacylglycerols (shown in Main Figure 2) from lipidomic analysis using targeted measurements. Data are presented as mean ± SEM. Statistical analysis was performed using repeated-measures two-way ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to control. Control (black) n=10, NAFLD (red) n=37.



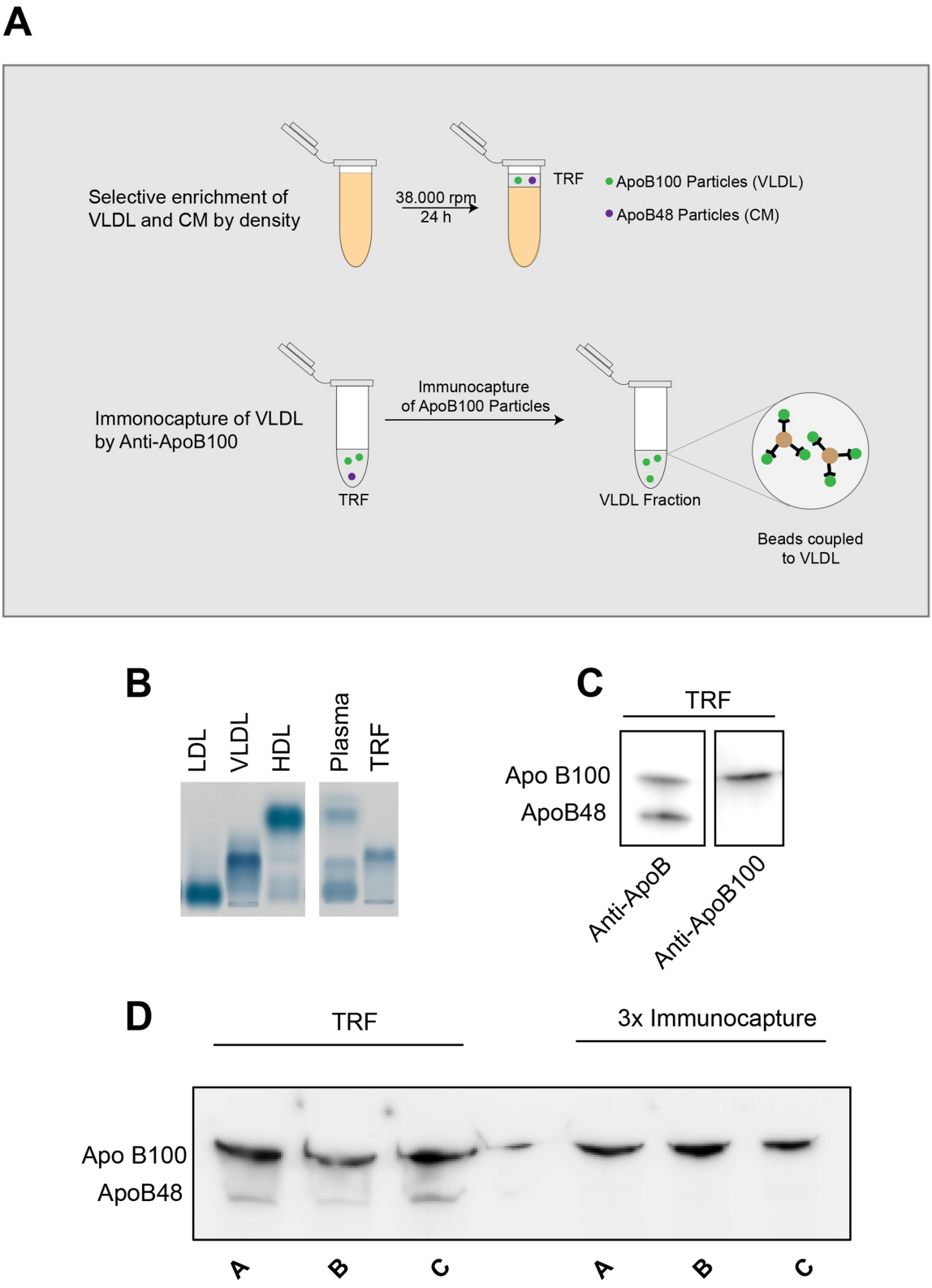
Supplemental Figure 2. Plasma diacylglycerol profiles follow a similar postprandial pattern in human NAFLD subjects. Relative abundance of additional postprandial plasma diacylglycerols profiled during targeted analysis. Data are presented as mean ± SEM. Statistical analysis was performed using repeated-measures two-way ANOVA, * P<.05, ** P<.01, *** P<.001, **** P<.0001 compared to control, Control (black) n=10, NAFLD (red) n=37.



Supplemental Figure 3. Most abundant plasma triacylglycerol species in postprandial plasma from human subjects. Relative abundance of the most abundant postprandial plasma triacylglycerols. Data are presented as mean ± SEM. Statistical analysis was performed using repeated-measures two-way ANOVA, * P<.05, ** P<.01, *** P<.001, **** P<.0001 compared to control. Control (black) n=10, NAFLD (red) n=37.

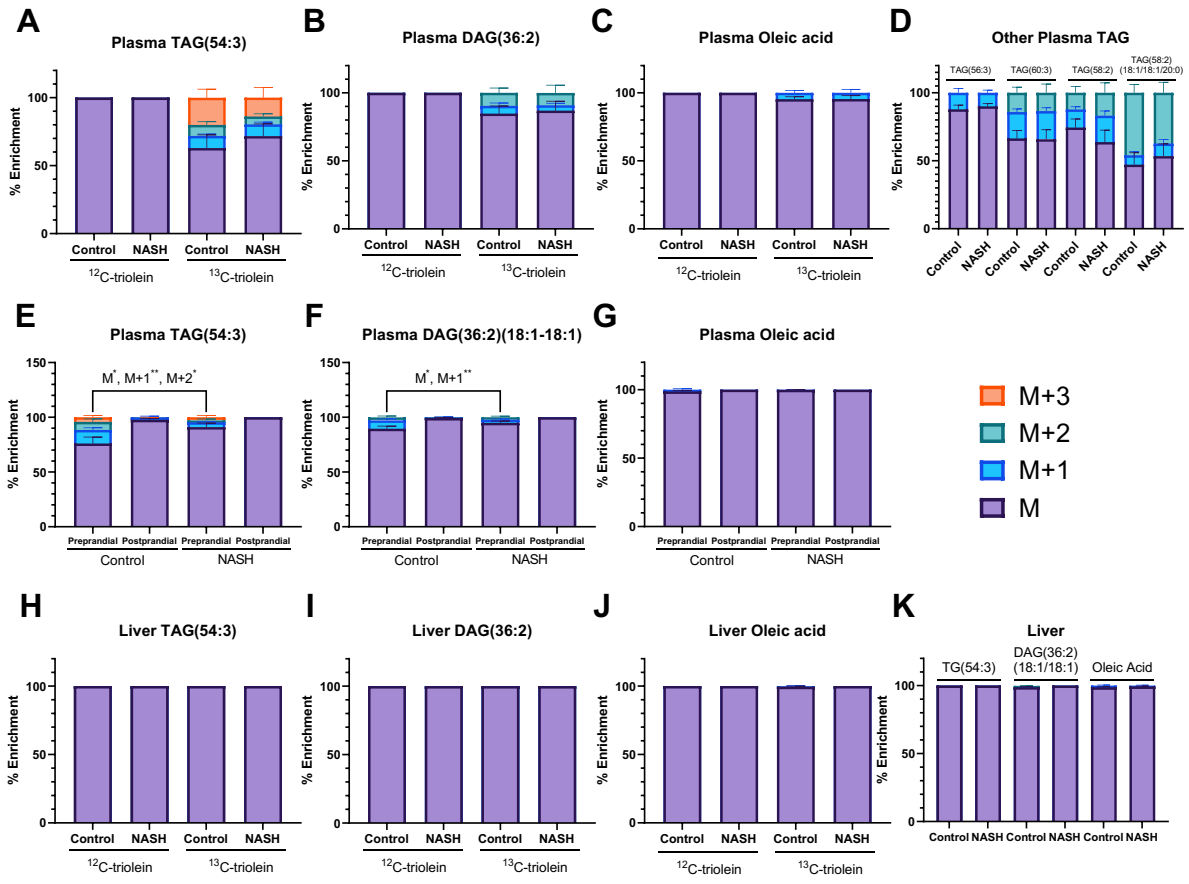


Supplemental Figure 4. Mice fed GAN-diet for 26 weeks develop NASH. Metabolic parameters of mice fed control diet or NASH-inducing GAN-diet for 26 weeks. (**A)** body weight; (**B**) fat mass; (**C**) lean mass; (**D**) representative H&E, oil red-o and Sirius red stained liver tissue; (**E**) liver weight; (**F**) liver index; (**G**) plasma alanine transaminase (ALT) and (**H**) aspartate transaminase (AST). Liver index is defined as liver weight divided by body weight. Body weight data is presented as mean ± SD, n=24-25. All other data is presented as the mean of each cage (4-5 mice) ± SEM, n=5.

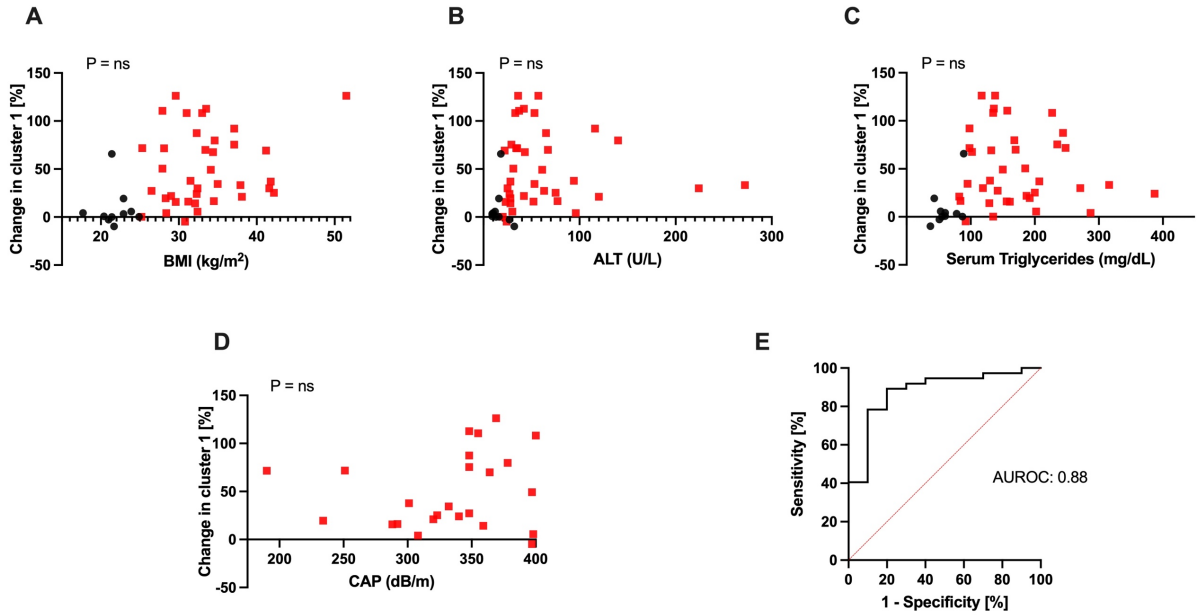


Supplemental Figure 5. **Isolation of VLDL and method validation.** (**A**) Schematic of the stepwise isolation of VLDL from postprandial plasma. (**B**) After differential centrifugation of plasma, the isolated triglyceride rich fraction (TRF) is selectively enriched

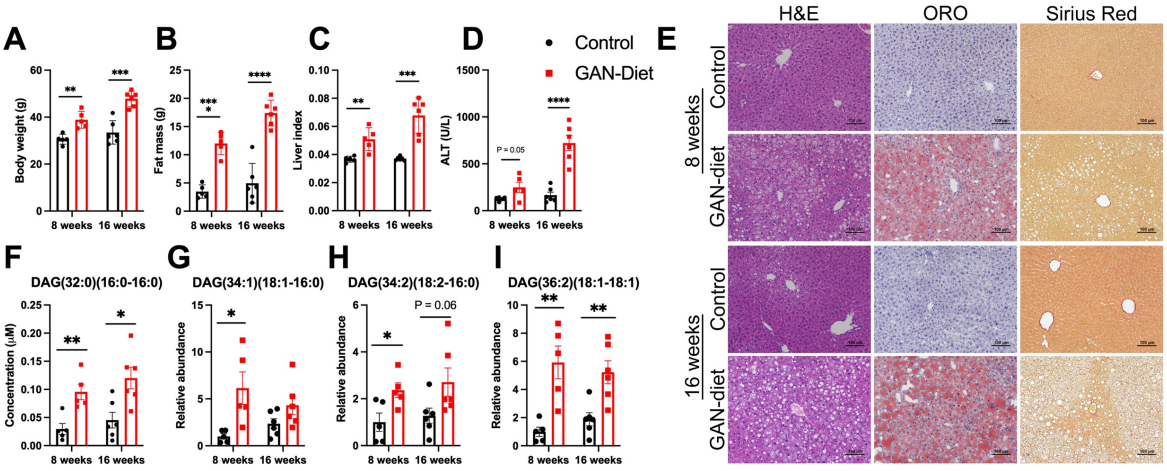
in low-density lipoprotein (VLDL) and chylomicrons (CM, no migration, band at origin) and does not contain low-density lipoprotein (LDL), or high-density lipoprotein (HDL) by gel electrophoresis. The anti-ApoB100 antibody is specific (**C**) and is able to selectively capture ApoB100 containing VLDL when bound to Dynabeads (**D**). D; n=3



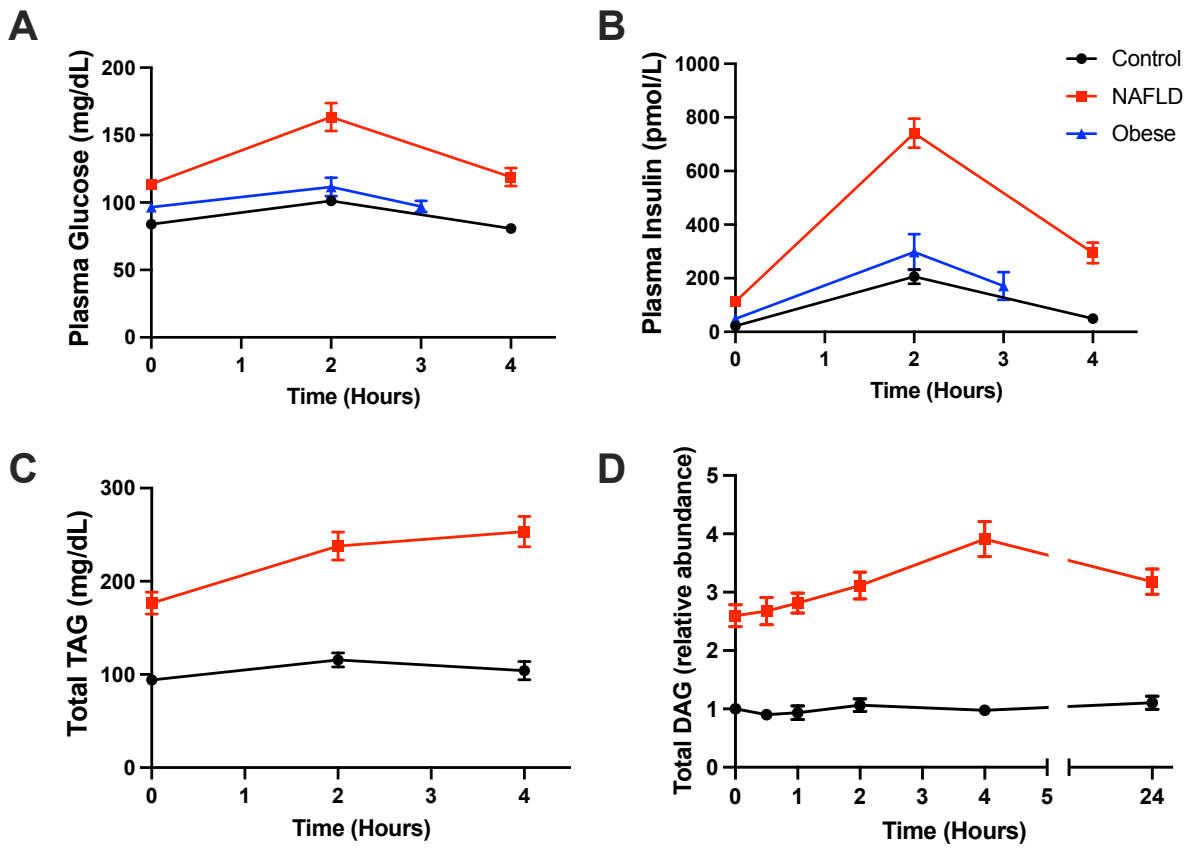
Supplemental Figure 6. Stable isotope enrichment of postprandial triacylglycerols, diacylglycerols and free fatty acids after administering ^{13}C-triolein in the current or previous meal. Postprandial plasma triacylglycerol (TAG(54:3)), diacylglycerol (DAG(36:2)), oleic acid as well as additional plasma TAGs with stable isotope enrichment when mice fed control and NASH diet for 26 weeks were given ^{13}C-triolein in the current (**A-D**) or previous (**E-G**) liquid meal. Postprandial liver triacylglycerol (TAG(54:3)), diacylglycerol (DAG(36:2)), oleic acid did not demonstrate detectable stable isotope enrichment when control and NASH mice were given ^{13}C-triolein in the current (**H-J**) or previous (**K**) liquid meal. Data are presented mean±SEM, n=5. All measurements occurred 1 hour postprandial unless otherwise stated. M denotes unlabeled lipid; M+1, a lipid with one labeled oleic acid; and M+2, a lipid with two oleic acids labeled, M+3, a lipid with three labeled oleic acids.



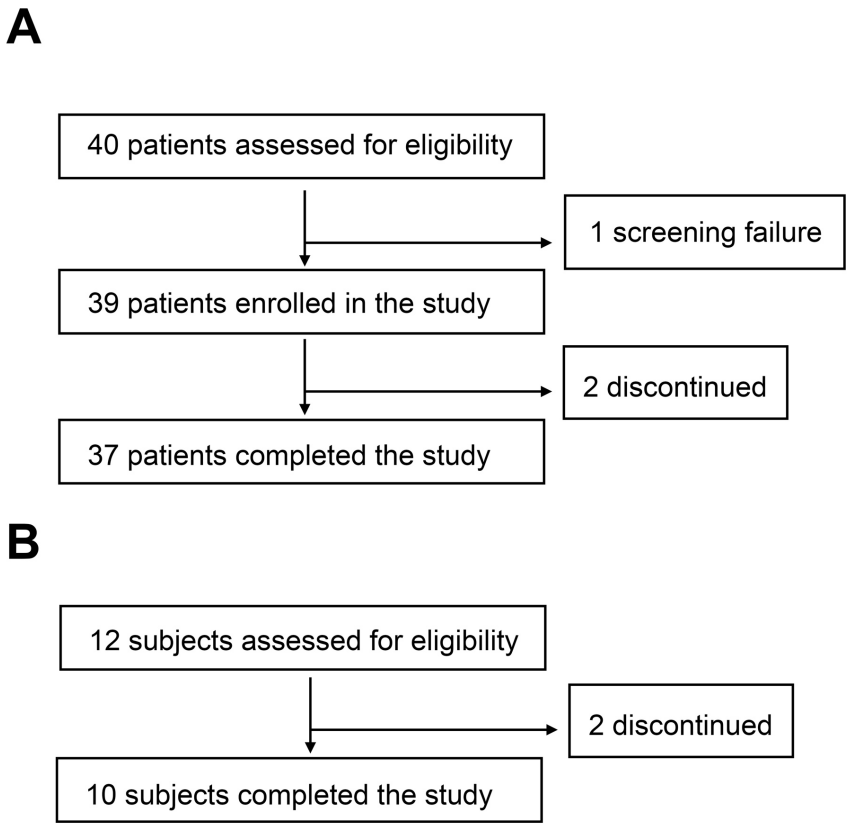
Supplemental Figure 7. Association of Cluster 1 and parameters of liver disease. Correlation between the relative change in Cluster 1 score and (**A**) Body Mass Index (BMI), (**B**) ALT, (**C**) serum triglyceride levels, and (**D**) Controlled Attenuation Parameter (CAP). Red – NAFLD, black – healthy controls. P-value from linear regression. (**E**) Receiver-operator characteristics (ROC) analysis of the ability of the 4-hour relative change in the Cluster 1 score to discriminate between NAFLD and controls



Supplemental Figure 8. Plasma postprandial diacylglycerols increase throughout murine NAFLD progression. Metabolic parameters of mice fed control diet or GAN-diet for 8- or 16-weeks. (**A**) body weight; (**B**) fat mass; (**C**) liver index; (**D**) plasma alanine transaminase (ALT); (**E**) representative H&E, oil red-o and Sirius red stained liver tissue; (**F-I**) postprandial plasma diacylglycerols in mice fed control (black) and GAN-diet (red) for 8- or 16-weeks. Mice were fasted for 6 hours and given a liquid meal bolus by gavage. Blood was collected 1 hour after gavage. Data are presented as mean ± SEM. Statistical analysis was performed by two-way ANOVA, * P<.05, ** P<.01. Liver index is defined as liver weight divided by body weight. Data are presented mean±SEM, n=5 (8 weeks), n=6 (16 weeks).



Supplemental Figure 9. Plasma metabolic parameters after a mixed meal. (**A**) Plasma glucose; (**B**) plasma insulin level; (**C**) total plasma triacylglycerols (TAG); (**D**) Total plasma diacylglycerols (DAG). Glucose and insulin levels measured at baseline, 2 hours and 4 hours postprandially (in controls and NAFLD subjects) or baseline, 2 hours and 3 hours (in non-NAFLD obese). Total TAG measured in controls and NAFLD subjects only. Total DAG (relative abundance) measured by summing abundance of all DAG species. Black – healthy controls, blue – non NAFLD obese, red – NAFLD. Mean±SEM.



Supplemental Figure 10. Study CONSORT diagram

Supplemental Tables

Supplemental Table 1 Histological diagnoses and scores in patients with available liver biopsy (n=15)

Feature	N (%)
NASH Diagnosis	13 (87%)
Steatosis[A]	
0	0 (0%)
1	7 (47%)
2	7 (47%
3	1 (7%)
Lobular Inflammation	
0	0 (0%)
1	9 (60%)
2	4 (27%)
3	2 (13%)
Hepatocyte Ballooning	
0	3 (20%)
1	9 (60%)
2	3 (20%)
Fibrosis	
0	3 (20%)
1	7 (47%)
2	2 (13%)
3	2 (13%)
4	1 (7%)
NAFLD Activity Score (NAS)	
Median	4
Range	2-7

[A]Histological scores based on the NASH-CRN scoring system

Supplemental Table 2. Identities of postprandial lipids found to be significantly different in Figure 1.

Cluster	Lipid Species	Identified Isomer(s)	ID Level	Class
1	DAG 34:1	DAG 16:0-18:1	2	DAG
1	DAG 34:2	DAG 16:0-18:2	2	DAG
1	DAG 36:1	DAG 18:0-18:1	2	DAG
1	DAG 36:2	DAG 18:1-18:1	2	DAG
1	DAG 36:3	DAG 18:1-18:2	1	DAG
1	DAG 36:4	DAG 18:2-18:2	1	DAG
1	DAG 36:4	DAG 18:1-18:3	2	DAG
1	DAG 38:3	DAG 18:2-20:1	2	DAG
1	DAG 38:5	DAG 16:0-22:5, DAG 18:1-20:4[A], DAG 18:2-20:3	2	DAG
1	PE 36:1	PE 18:0-18:1	2	PE
1	PI 38:4	PI 18:0-20:4	2	PI
1	TAG 54:9	TAG 18:3-18:3-18:3,	2	TAG
1	TAG 56:10	TAG 16:0-18:3-22:7, TAG 16:0-18:4-22:6, TAG 16:0-20:4-20:6 TAG 16:0-20:5-20:5, TAG 16:1-18:3-22:6, TAG 16:1-20:4-20:5, TAG 16:2-20:4-20:4	2	TAG
2	CE 20:4	CE 20:4	2	CE
2	CE 20:5	CE 20:5	2	CE
2	CE 22:6	CE 22:6	2	CE
3	ACar 14:0	ACar 14:0	2	ACar
3	ACar 18:1[B]	ACar 18:1	2	ACar
3	ACar 18:2[B]	ACar 18:2	2	ACar

[A]Most abundant isomer in targeted analysis
[B]Lipid species with two lipid features in Cluster due to m/z binning leak

Supplemental Table 3. Demographic and clinical characteristics of obese non-NAFLD cohort (n=12)

Age (years)	48.3 (11.5)
Sex – (female %)	11 (92%)
Ethnicity [n (%)]	
Caucasian	7 (58.3%)
Hispanic	1 (8.3%)
African-American	4 (33.3%)
BMI (kg/m^2)	37.9 (12.4)
Fasting Insulin (mcU/mL)	8.0 (5.7)
Fasting glucose (mg/dL)	97 (6.9)
HOMA-IR	1.9
Triglycerides (mg/dL)	105 (59)
Mixed Meal Dose (kCal)	360

Demographic and clinical characteristics are presented as mean (SD) unless otherwise stated.

Supplemental Table 4. Multiple reaction monitoring (MRM) transitions for targeted diacylglycerol analysis

Lipid	Q1 Mass (Da)	Q3 Mass (Da)	Retention Time (min)
DAG(36:3)(18:1-18:2)	636.5	339.3	2.34
DAG(36:4)(18:2-18:2)	634.6	337.2	2.04
DAG(36:5)(18:1-18:3)	632.5	337.2	1.82
DAG(34:1)(18:1-16:0)	612.5	339.3	2.62
DAG(34:2)(18:1-16:1)	610.5	313.3	2.31
DAG(36:1)(18:0-18:1)	640.6	341.3	3.04
DAG(36:2)(18:1-18:1)	638.6	339.3	2.64
DAG(36:4)(18:1-18:3)	634.6	335.2	2.04
DAG(38:5)(20:4-18:1)	634.6	335.2	2.21
Ceramide (d18:1/17:0) (IS)	552.7	264.3	2.14

IS, internal standard