SUPPLEMENTARY METHODS for the article entitled
"Elevated de novo lipogenesis is a distinctive characteristic of individuals with nonalcoholic fatty liver disease"

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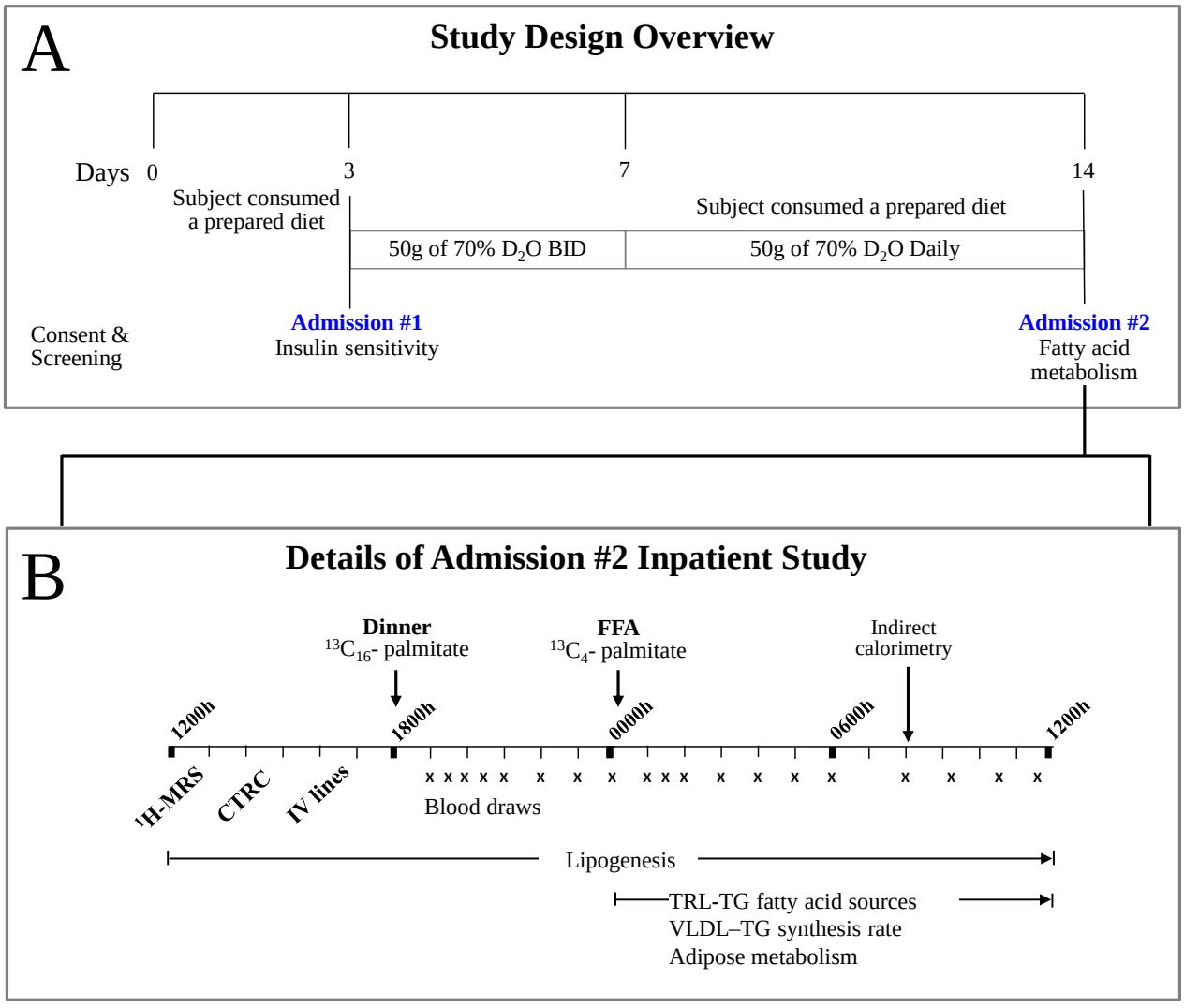
Appendix Figure 1. Schematic representation of the overall study design (A) and the inpatient studies (B)



(A) Each subject participated in two metabolic tests - the first designed to assess insulin sensitivity (Admission #1) and the second designed to measure fasting fatty acid metabolism (Admission #2). During admission #1, subjects underwent a frequently-sampled insulin-modified IVGTT (described in the text). **(B)** During Admission #2, subjects were studied over an 18h period for measurement of fatty acid flux from DNL, dietary fat, and adipose-derived fatty acids in plasma FFA and VLDL-TG.

Subjects

Based on the results of the initial screen, eligible subjects were invited to attend a more comprehensive screening to 1) assess characteristics of the metabolic syndrome (elevated plasma TG, waist circumference, and low HDL-cholesterol (1) , 2) obtain a medical history and weight history (loss/gain) and 3) rule out diabetes (fasting glucose concentrations <126 mg/dL, HbA1c <6.5%) and pre-existing liver disease including hepatitis, cirrhosis, biliary atresia, cholestasis, and genetic disorders. Subjects were excluded if they smoked, had known metabolic abnormalities including elevated thyroid hormone levels, used dietary supplements beyond multivitamins, or had unusual eating habits (dietary restrictions, very low-fat diets). Subjects were also interviewed by a registered dietitian regarding frequency and quantity of alcohol intake, and were excluded if they consumed >140 g/week for men and >70 g/week for women.

Following the second screening visits, 24 subjects were willing to participate in the research protocol, which required controlled food intake and activity on an outpatient basis, multiple inpatient visits with isotopes administered via IV and the diet, and an ^{1}H-MRS to determine liver fat content (2). The sample size was powered to assess lipogenesis and detect metabolic differences in high vs. low liver-fat subjects (3-5).

Once entered into the study, each subject participated in two in-patient tests (**appendix fig. 1**) - the first (admission #1) designed to assess insulin sensitivity using a frequently-sampled, insulin-modified intravenous glucose tolerance test (IVGTT) (6) and the second (admission #2) designed to measure fatty acid metabolism. Prior to admission #1, subjects completed a 3d food intake diary and a 24h dietary recall and interview with a registered dietitian to

determine usual dietary intake. Food records were analyzed using the Nutrition Data System for Research (NDSR 2009; Minneapolis, MN) and using this information, the 24h recall and indepth interview, a weight-maintaining menu based was designed by the dietitian, prepared by the Clinical and Translational Research Center (CTRC) kitchen. The weight-maintaining diet including all foods and beverages was given to the subjects to consume on an outpatient basis for 3d prior to admission #1 and 7d prior to admission #2. No alcohol was consumed for 3d before each admission. Subjects had stable body weight and maintained pre-enrollment physical activity before and during the two-week testing period.

Testing timelines

For admission #1, subjects reported to the CTRC at 0700 to undergo the IVGTT, as described in detail previously (6). Glucose and insulin responses were analyzed using the minimal model technique and MINMOD Millenium software (7). Body composition was determined by DEXA (Hologic Discovery W, QDR series; Bedford, MA) and intrahepatic-TG was measured by 3.0 Tesla ^{1}H-MRS (2). Subjects wore an Actical® accelerometer at hip level (Actical®; V2.1; Philips Healthcare; Andover, MA) for 4d to assess physical activity levels (9). METs were calculated based on counts per minute of activity and sedentary activity corresponded to 0-1 MET, light activity 1-3 MET, and moderate activity 3-6 MET (9).

The last day of admission #1, the subjects were given 3 loading doses of D_2O to bring body water enrichments to at least 0.6%. After this, they consumed 50g doses of 70% deuterated water (D_2O; Cambridge Isotope Laboratories, Inc.; Andover, MA) twice per day (BID) for 3d and once per day for the final 7d before admission #2 (**appendix fig. 1**) (10). This duration of D_2O administration was chosen to improve the assessment of hepatic de novo lipogenesis

(DNL) in subjects with fatty liver (larger intrahepatic TG pool sizes) compared to overnight D_2O administration or 10d of acetate infusion, as we have performed previously (4). After the 10d of dosing, body water D_2O enrichments averaged 0.86 ± 0.18% in the subjects (Metabolic Solutions Inc., Nashua, NH).

To begin admission #2, each subject was admitted to the CTRC and IV lines were placed. At 1800h, the subject consumed a standardized evening meal consisting of solid foods (banana and cereal with skim milk) and a beverage (cocoa, corn oil, heavy cream, sucrose, and skim milk) containing 1g of U-^{13}C-palmitate (Isotec/Sigma, Miamisburg, OH) to trace meal fat absorption and incorporation into lipoprotein-TG, as well as spillover of dietary fatty acids into the plasma FFA pool (11). This meal provided 38% of total daily energy needs and the composition was 831 ±131 kcal, 42.4 ± 2.6 g fat, 17.3 ± 6.7 g protein, and 103.6 ± 25.4 g carbohydrate (analyzed using NDSR). Subjects remained fasted after the meal, and the next morning breakfast was withheld to allow for complete turnover of the plasma VLDL-TG pool (18h since the last meal). At midnight an IV infusion of [1,2,3,4]-$^{13}C_4$-palmitate (7 µg/kg/min; Isotec; St. Louis, MO) was initiated to measure 1) the contribution of plasma FFA to hepatic-TG synthesis (11), 2) the rate of appearance of adipose FFA and 3) the rate of appearance of dietary FA spillover (see below). Fasting indirect calorimetry was performed from 0800 to 0830 on day 2 using a metabolic cart (VMax Encore, Viasys Healthcare; San Diego, CA) and energy expenditure, fat and glucose oxidation were calculated using standard equations (12), with protein catabolism calculated based on the NDSR-calculated protein intake from the previous day.

Laboratory procedures and fatty acid analysis

From 1800 on day 1 through noon on day 2 of admission #2, blood was collected at 26 time points and plasma immediately separated, kept on ice, and a preservation cocktail of PMSF, chloramphenicol, gentamicin sulfate, benzamidine and Trolox was added (11). Plasma FFA, TG, and glucose concentrations were measured enzymatically (kit #991-34891, #998-40391/994-40491, and #439-90901, respectively, Wako Diagnostics, Richmond, VA). Insulin was measured by ELISA (#EZHI-14L, Millipore Corporation, Billerica, MA) and apoB48 (AKHB48E; Shibayagi Co., Ltd., Biovendor LLC, Asheville, NC) was measured by ELISA. Due to volume limitations on the amount of blood drawn, at 14 of these time points (from midnight until noon), total TG-rich lipoproteins (TRL) were isolated from plasma by fixed-angle ultracentrifugation at 40,000 RPM for 20h in a 50.3Ti rotor (Beckman Instruments; Palo Alto, CA) at 15°C (11). After lipoprotein isolation from plasma, TG was separated from TRL and TG-fatty acids were prepared for GC/MS as described previously (11). FFA from plasma were also prepared for GC/MS as described (13). The labeling patterns of TG and FFA were determined by measurement of isotopic enrichments on an Agilent 6890N GC coupled to a 5975 MS detector and 7683B injector (Agilent Technologies, Palo Alto, CA) (5). Enrichments were determined by selective ion monitoring for m/z of 270, 271, 272, 274, and 286 under electrical ionization using 4-6 point standard curves. The different species of fatty acids in lipoprotein-TG and plasma FFA were measured by GC with flame ionization detector (14).

Calculations and statistical analysis

TG- fatty acid sources

The identity of fatty acid sources contributing to TG synthesis were calculated using our previously-established, multiple stable-isotope procedure (4, 5, 11, 13, 15, 16). Briefly, the

potential sources of fatty acids that contribute to lipoprotein-TG production (dietary fat, adipose fatty acids, and hepatic de novo lipogenesis) are each labeled with a different isotope. Palmitate serves as the marker for all fatty acids because it is the primary product of de novo lipogenesis and it is the second most prominent fatty acid in the diet. Here, the contributions of the fatty acid sources are presented first as a proportion of TRL-TG fatty acids, which reflects the flux of fatty acids into intracellular-TG synthesis (4, 17-19) as demonstrated in liver biopsy studies previously published by us (4) and others (20, 21). The proportions of palmitate derived from each source are then multiplied by the absolute concentrations of TRL-TG palmitate to determine the quantitative contributions of these sources to TG-palmitate concentrations. Finally, these values are divided by the percentage of TRL-TG fatty acids that are palmitate to reflect all TG-fatty acids derived from each source. It is possible that not all fatty acid sources contributing to VLDL-TG will be identified using these three stable isotopes, as we have demonstrated previously (4, 15). The fatty acids remaining unlabeled at the end of the 18h study could be derived from visceral stores or from intrahepatic-TG droplets (22).

The plasma FFA pool can be diluted by spillover of dietary fatty acids, therefore a correction is applied to the calculation of FFA arising from adipose tissue to account for FA that do not arise from adipose lipolysis (i.e. that instead arise from dietary spillover) (11). Mass isotopomer distribution analysis was used to calculate the percentage of newly made palmitate in VLDL (11). DNL was calculated from samples throughout the evening (from midnight to noon). Fasting DNL was defined as the percent contribution of newly synthesized FA in the VLDL-TG pool expressed as an average of the last two blood samples taken (10:30 and 11:45 in the morning). Due to the lengthy labeling period (10d), DNL-derived FA in

VLDL-TG may arise from newly synthesized FA in the liver (as well as previously synthesized FA that has been stored in IHTG), FA synthesized in adipose tissue, and DNL-FA that have been exported in VLDL-TG and entered the plasma FFA pool through lipolysis of either VLDL particles or stored adipose-TG. To reduce the potential over-accounting of hepatically-derived DNL-FA, the proportion of DNL-FA in the fasting plasma FFA pool was measured (average ± SD, 5.7% ± 4.7%) and taken into consideration in the calculation of the proportion of DNL-FA in VLDL-TG.

The model of VLDL-TG production rate

Fractional VLDL-TG turnover rate was calculated by modeling the rise to plateau enrichment of [1,2,3,4]-^{13}C-palmitate into lipoprotein-TG using SigmaPlot (V12.0; Systat Software Inc., San Jose, CA) (11). This model assumes that the plasma FFA label, when used for lipoprotein-TG synthesis occurs almost entirely in the liver. Although there are some data in rodents that suggest the intestine can take up plasma FFA and use them for TG synthesis, this has not been demonstrated in humans. Further, our previous data from fasting and fed humans do not support this concept. We have found that an IV infusion during the fasting state will label TG in both large TRL which are VLDL with minute amounts of chylomicrons produced in the fasting state, and small TRL, which are again, primarily VLDL particles. However, as we continue to infuse this label during the fed-state, the label's presence drops out of the buoyant particle fraction (Sf >400), and this buoyant fraction represents primarily chylomicrons. For detailed illustration of these observations, please refer to the figure shown at the end of this document (**appendix figure 2**).

These data demonstrate that even in a mixed fraction of TRL particles (containing both chylomicrons and VLDL) the IV-infused $^{13}C_4$-palmitate is utilized for VLDL-TG synthesis. In the present study, we started collecting samples for analysis of VLDL-TG production rate at midnight. However, at this time, the TRL fraction contains both chylomicrons and VLDL. As the infusion continues throughout the early morning hours, the subject becomes more fasted and the apoB48 concentrations of this fraction decay to low levels between 0300-0600; thereafter isolated TRL contain VLDL. At the end of the measurement (1200 noon, or 18h after the last meal), the TRL fraction represents VLDL, a commonly made assumption by nearly all investigators in the field, even though some chylomicrons can still be present in the fasting state. Using this information we modeled the $^{13}C_4$-label in the TRL throughout this period to obtain production rate data for VLDL-TG. On the other hand, we used the final fractional sources of palmitate at the end of the experiment to determine the contribution of FA sources from diet, adipose and de novo lipogenesis to VLDL-TG synthesis at steady state.

Plasma FFA sources

Sources of plasma FFA include 1) the adipose tissue, which supplies the majority of FFA in the fasting state, and 2) dietary fatty acids, liberated from chylomicrons undergoing lipolysis in the plasma via the enzyme lipoprotein lipase – the so-called spillover pathway (11) (38,39).

The *rate of appearance* of adipose fatty acids (Adipose RaFFA) was calculated using standard dilution equations (24) which have been corrected for the presence of dietary spillover (as above) (11). A standard set of assumptions was used for the calculations: (1) serum volume was 4.5% total body weight, (2) palmitate served as a marker for all other FA in the diet and plasma, and (3) the incorporation of the plasma FFA label into VLDL-TG was

used to calculate VLDL-TG production rate (11). Fasting RaFFA was determined as the average between the 1030 and 1145 time points.

The IV infusate was measured by GC and GC/MS to determine the FA composition and the amount of unlabeled FA present from the albumin solution used in the infusion (11). The calculations for RaFFA were adjusted for unlabeled FA occurring in the infusate solution (11). The amount of FA arising from spillover of meal-fat was also determined to correct for contribution of these FA in order to determine FFA arising from adipose-TG lipolysis alone (11). The composition of plasma FFA was determined by GC and the percentage of palmitate used to determine total RaFFA (11).

The *quantity* of FFA arising from spillover was determined by the presence of the dietary label (U-^{13}C-palmitate) found in the FFA pool (11). Knowing these data, in combination with measured meal palmitate enrichments, unlabeled dietary fatty acids which have also spilled over to produce FFA were accounted for in the calculation of total dietary fatty acid spillover (11). We have extended this method to calculate the *rate of appearance of spillover fatty acids* (RaSpillover) using the known rate of IV infusion of [1,2,3,4]-^{13}C-palmitate and comparing the proportion of this tracer in the plasma FFA pool to that of the dietary fatty acids. This calculation is as follows:

$$\text{RaSpillover}\ \left(\frac{\mu\text{mol}}{\text{min}}\right) = \frac{\text{Ri}_{\text{13C4-Palm}}\ \left(\frac{\mu\text{mol}}{\text{min}}\right)}{\left[\frac{\text{FFA}_{\text{13C4-Palm}(\%)}}{(\text{FFA}_{\text{13C4-Palm}\ (\%)} + \text{FFA}_{\text{spillover}\ (\%)})}\right]} - \text{Ri}_{\text{13C4-Palm}}\ \left(\frac{\mu\text{mol}}{\text{min}}\right)$$

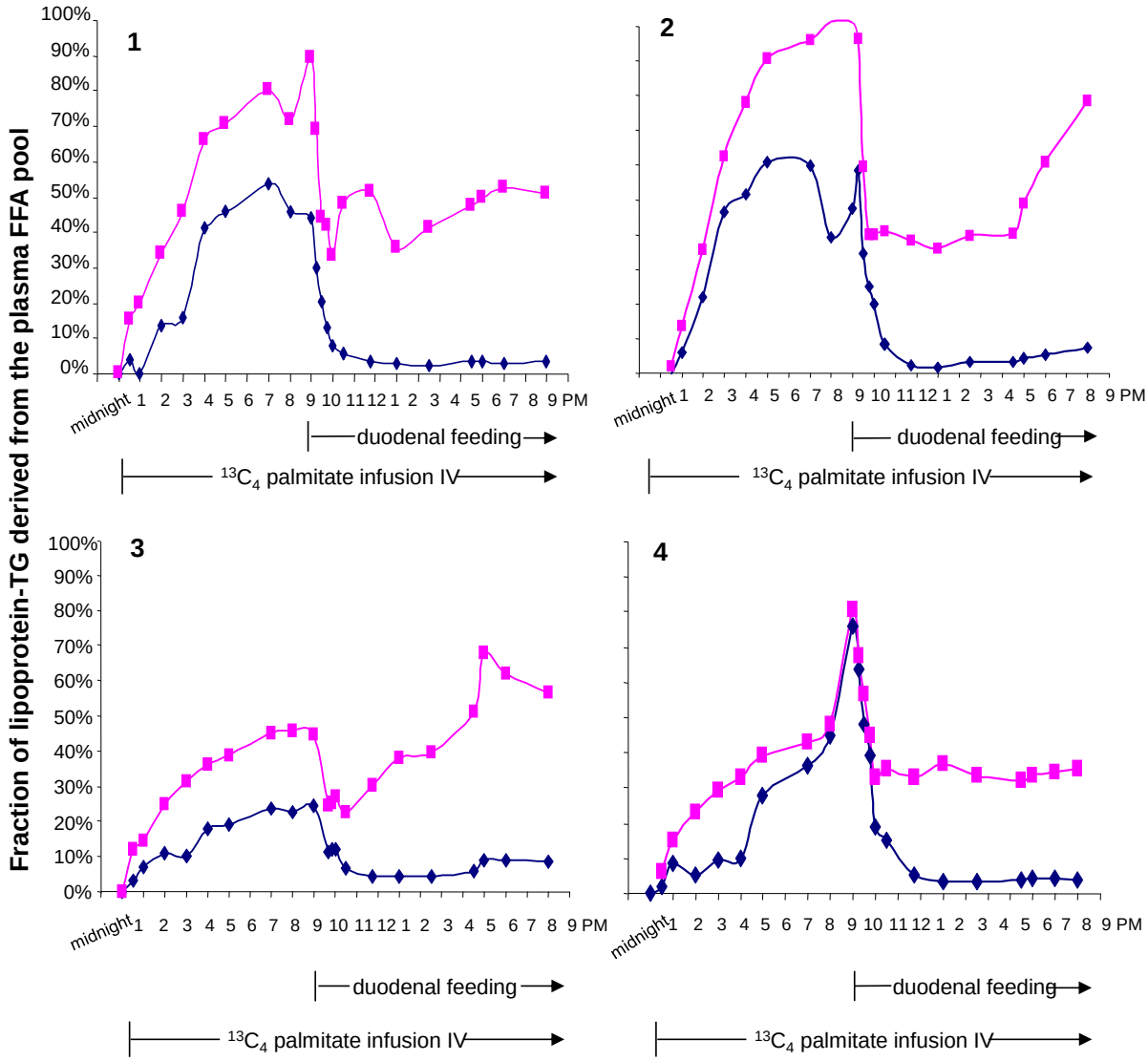
where $\text{Ri}_{\text{13C4-Palm}}$ = infusion rate of [1,2,3,4]-^{13}C-palmitate; $\text{FFA}_{\text{13C4-Palm}\ (\%)}$ = enrichment of [1,2,3,4]-^{13}C-palmitate in the plasma FFA pool; and $\text{FFA}_{\text{Spillover}\ (\%)}$ = proportion of total dietary-derived fatty acids (labeled and unlabeled) in the plasma FFA pool. Adipose RaFFA and

RaSpillover were determined at each time point from midnight to noon on day 2. The composition of plasma FFA was determined by GC and the percentages of palmitate were used to determine total RaFFA, Adipose RaFFA (11), and RaSpillover.

Appendix Figure 2. Presence of $^{13}C_4$-palmitate, infused IV, as it appears in more buoyant (S_f >400) and less buoyant (S_f 60-400) TG-rich lipoproteins during the fasted and fed states

Plasma FFAs moving into TGs in lipoproteins with S_f 60-400 and S_f > 400

Interpretation: *In the fasting state*, the plasma FFA label was used to synthesize both small (S_f 60-400) and large (S_f >400) VLDL particles (apoB48 concentrations were below the detection limit in these fractions). By contrast, *in the fed state* (starting at 9h), the FFA label became non-detectible in the S_f >400 fraction (containing apoB48) and no apoB48 was detected in the S_f 60-400 fraction.



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