Reproducibility of stable isotope-labeled tracer measures of VLDL-triglyceride and VLDL-apolipoprotein B-100 kinetics

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Abstract To gain insight into the mechanisms regulating plasma lipid homeostasis, FFA, VLDL-triglyceride (TG), and VLDL-apolipoprotein B-100 (apoB-100) kinetics are commonly assessed using stable isotope-labeled tracer methods. The reproducibility of these measurements, which is critical for the experimental design, is unknown. Therefore, we investigated the repeatability of plasma FFA, VLDL-TG, and VLDL-apoB-100 kinetics in eight healthy men using stable isotope-labeled tracer techniques. There were no systematic differences in plasma FFA, VLDL-TG, and VLDL-apoB-100 concentrations and kinetics between the two studies. Intra-individual day-to-day variability for various outcome variables ranged from 15% to 25%, and almost all of this was of biological origin. The most robust outcome variables were FFA rate of appearance and hepatic VLDL-TG and VLDL-apoB-100 secretion rates; the least robust were VLDL-TG and VLDL-apoB-100 plasma clearance rates and mean residence times. Overall, physiologically meaningful differences in mean values (i.e., 25–30% in magnitude) can be obtained with a sample size of 6–10 subjects for paired studies and 12–20 subjects per group for cross-sectional studies, assuming a type I error rate of 0.05 and a type II error rate of 0.20 (i.e., 80% power). These findings will be useful for future studies investigating FFA, VLDL-TG, and VLDL-apoB-100 kinetics with the methods described.—Magkos, F., B. W. Patterson, and B. Mittendorfer. **Reproducibility of stable isotope-labeled tracer measures of VLDL-triglyceride and VLDL-apolipoprotein B-100 kinetics.** *J. Lipid Res.* 2007. **48:** **1204–1211.**

VLDL is a spherical, macromolecular complex of apolipoproteins and lipids. The major components of VLDL, the structural apolipoprotein B-100 (apoB-100) and core triglyceride (TG), are produced by the liver, assembled into VLDL, and secreted into the bloodstream. Circulating VLDLs exchange TG for cholesteryl esters with HDL and act as acceptors of surface apolipoproteins (e.g., apoC-II and apoE from HDL); VLDLs also interact with LPL in the capillary beds of adipose tissue and cardiac and skeletal muscle. LPL hydrolyzes the bulk of TG in VLDL, thereby releasing FFA for storage or energy production. Stepwise delipidation of VLDL results in the formation of VLDL remnants and intermediate density lipoproteins; these are taken up by the liver or further depleted of TG through the action of LPL and hepatic lipase, which converts the particle to LDL. Therefore, VLDL is important for plasma lipid homeostasis. Dysregulation of VLDL metabolism is associated with a proatherogenic plasma lipid profile (i.e., increased plasma TG concentration, decreased HDL-cholesterol and increased LDL-cholesterol concentrations, increased VLDL and/or LDL particles in the circulation) and increased cardiovascular disease risk (1, 2). Understanding VLDL-TG and VLDL-apoB-100 metabolism, therefore, has important physiological and clinical implications.

A variety of tracer techniques are available for evaluating VLDL-TG and VLDL-apoB-100 kinetics (3–8). Most contemporary methods make use of stable isotope-labeled tracers to measure the rate of incorporation and/or loss of the tracer into TG or apoB-100 in VLDL, often in combination with compartmental modeling analysis. The duration of the experimental protocol (i.e., several hours) and possible confounding attributable to tracer recycling make it impossible to study the acute effects of metabolic perturbations (e.g., hyperinsulinemia, exercise, etc.) in a single session and require that subjects be studied on two different occasions several days or weeks apart. Owing to tracer cost and analytical burden, studies are usually conducted in small samples, typically 5–10 subjects (9–19). The design and interpretation of these studies (particularly when dealing with negative findings) is difficult because there are no data available on the reliability of tracer measures of VLDL-TG and VLDL-apoB-100 kinetics. Therefore, we

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determined the reproducibility and degree of intraindividual variability of basal VLDL-TG, VLDL-apoB-100, and plasma FFA [a major precursor for VLDL-TG synthesis (10, 11, 20)] kinetics, assessed using stable isotope-labeled tracer methods. We then used this information to provide estimates of minimum detectable differences for any given sample size when assessing FFA, VLDL-TG, and VLDL-apoB-100 metabolism with the methods described here.

MATERIALS AND METHODS

Subjects

Eight men [age, 30 ± 6 years; body weight, 76 ± 6 kg; body mass index, 23 ± 2 kg/m^2; body fat, assessed by dual-energy X-ray absorptiometry (Delphi-W densitometer; Hologic, Waltham, MA), 16 ± 5% (means ± SD)] participated in this study. All subjects were considered to be in good health after completing a medical evaluation, which included a history and physical examination and standard blood tests. None of the subjects smoked or took medications for at least several months before and during the study. Written informed consent was obtained from all subjects before their participation in the study, which was approved by the Human Studies Committee and the General Clinical Research Center (GCRC) Advisory Committee at Washington University School of Medicine in St. Louis, MO.

Experimental protocol

To determine plasma FFA, VLDL-TG, and VLDL-apoB-100 kinetics, each subject completed two identical stable isotope-labeled tracer infusion studies (≥2 weeks apart) after an overnight fast. Subjects were instructed to adhere to their regular diet and to refrain from exercise for 3 days before each study. They were admitted to the GCRC on the afternoon before the tracer infusion study. At 7:30 PM, they consumed a standard meal containing 12 kcal/kg body weight with 55% of total energy provided as carbohydrates, 30% as fat, and 15% as protein. Subjects then fasted (except for water) and rested in bed until completion of the study the next day. At 5:30 AM the following morning, one catheter was inserted into a forearm vein to administer stable isotope-labeled tracers; a second catheter was inserted into a vein in the contralateral hand, which was heated to 55°C with a thermostatically controlled box, to obtain arterialized blood samples. Catheters were kept open with slow, controlled infusion of 0.9% NaCl solution (30 ml/h). At 7:00 AM (time 0), after blood samples for the determination of plasma substrate and hormone concentrations and background glycerol, palmitate, and leucine tracer-to-tracee ratios (TTRs) in plasma and VLDL-TG and apoB-100 were obtained, a bolus of [1,1,2,3,3-^{2}H$_5$]glycerol (75 μmol/kg), dissolved in 0.9% NaCl solution, was administered through the catheter in the forearm vein, and constant infusions of [2,2-^{2}H$_2$]palmitate (0.03 μmol/kg/min), dissolved in 25% human albumin solution, and [5,5,5-^{2}H$_3$]leucine (0.12 μmol/kg/min; priming dose, 7.2 μmol/kg), dissolved in 0.9% NaCl solution, were started and maintained for 12 h. Blood samples were collected at 5, 15, 30, 60, 90, and 120 min and then every hour for 10 h to determine glycerol and palmitate TTRs in plasma and VLDL-TG and leucine TTR in plasma and VLDL-apoB-100.

Sample collection and storage

To determine plasma glucose concentration, blood was collected in tubes containing heparin; plasma was separated by centrifugation and analyzed immediately. All other blood samples were collected in chilled tubes containing sodium EDTA. Samples were placed on ice, and plasma was separated by centrifugation within 30 min of collection. Aliquots of plasma were kept in the refrigerator for immediate isolation of VLDL and measurement of plasma apoB-100 concentration. The remaining plasma samples were stored at −80°C until final analyses were performed.

The VLDL fraction was prepared as described previously (13). Briefly, ∼1.5 ml of plasma was transferred into OptiSeal polyallomer tubes (Beckman Instruments, Palo Alto, CA), overlaid with a NaCl/EDTA solution (1.006 g/ml), and centrifuged at 100,000 g for 16 h at 10°C in an Optima LE-80K preparative ultracentrifuge equipped with a type 50.4 Ti rotor (Beckman Instruments). The top layer, containing VLDL, was removed by tube slicing (CentriTube slicer; Beckman Instruments). Aliquots of the VLDL fraction were set aside for measuring VLDL-apoB-100 concentration immediately after collection; the remaining samples were stored at −80°C until final analyses were performed.

Substrate and hormone concentrations

Plasma glucose concentration was determined on an automated glucose analyzer (YSI 2300 STAT plus; Yellow Springs Instrument Co., Yellow Springs, OH) (21, 22). Plasma insulin concentration was measured by RIA (Linco Research, St. Louis, MO) (23, 24). Plasma FFA concentrations were quantified by gas chromatography (HP 5890 series II gas chromatograph; Hewlett-Packard, Palo Alto, CA) after adding heptadecanoic acid to plasma as an internal standard (25). Total plasma TG and VLDL-TG concentrations were determined with a colorimetric enzymatic kit (Sigma Chemical Co., St. Louis, MO) (26). Total plasma apoB-100 and VLDL-apoB-100 concentrations were measured with a turbidimetric immunoassay (Wako Pure Chemical Industries, Osaka, Japan) (27).

Substrate enrichments

Plasma free glycerol, palmitate, and leucine TTRs, the TTRs of glycerol and palmitate in VLDL-TG, and the TTR of leucine in VLDL-apoB-100 were determined by electron-impact ionization GC-MS (Agilent Technologies/HP 6890 Series GC System and 5973 Mass Selective Detector; Hewlett-Packard) (7, 9, 13, 25, 28). To determine plasma free glycerol, palmitate, and leucine TTRs, plasma proteins were precipitated with ice-cold acetone, and the supernatant was collected, mixed with equal volumes of water and hexane, and centrifuged to separate the solvent and aqueous phases. The bottom phase (water), containing glycerol and leucine, was dried under vacuum. Glycerol TTR was determined by forming the heptafluorobutyryl (HFB) derivative and selectively monitoring ions at m/z 253 (m+0) and 257 (m+4) by GC-MS. Leucine TTR was determined by forming the tertiary-butyldimethylsilyl derivative and selectively monitoring ions at m/z 200 (m+0) and 203 (m+3) by GC-MS. The upper phase (hexane), containing plasma lipids, was dried under vacuum, and fatty acids were converted to their methyl esters (FAMEs) with iodomethane. FAMEs were extracted with hexane and isolated by solid-phase extraction column chromatography. Samples were dried under vacuum and reconstituted with heptane, and ions at m/z 270 (m+0) and 272 (m+2) were analyzed by GC-MS for plasma palmitate TTR.

To determine glycerol and palmitate TTRs in VLDL-TG and leucine TTR in VLDL-apoB-100, the VLDL fraction was treated with n-butanol-isopropyl ether to selectively precipitate VLDL-apoB-100 at the interface between the aqueous and solvent phases; the top and bottom layers containing VLDL lipids and soluble apolipoproteins were combined, mixed with chloroform-methanol, and centrifuged, and the bottom layer containing VLDL lipids was dried under vacuum. VLDL-TGs were isolated by

TLC, extracted with chloroform-methanol, and dried under vacuum. FAMEs were formed during incubation with acetyl chloride in methanol and dried under vacuum; the glycerol liberated by the transmethylation reaction was derivatized by adding HFB anhydride in ethyl acetate. Samples were dried under vacuum and reconstituted with heptane. The TTRs of HFB-glycerol and palmitate methyl ester were determined by selectively monitoring ions at m/z 467 (m+0) and 472 (m+5) for VLDL-TG glycerol and at m/z 270 (m+0) and 272 (m+2) for VLDL-TG palmitate by GC-MS. VLDL-apoB-100 was hydrolyzed during incubation with HCl and dried under vacuum. Amino acids were partially derivatized during incubation with acetyl chloride in propanol, dried under vacuum, and subsequently incubated with HFB anhydride in ethyl acetate to form the N-heptafluorobutyryl n-propyl ester derivative. Saturated saline and heptane were used to extract derivatized amino acids (heptane phase). After drying under vacuum, samples were reconstituted with heptane, and ions at m/z 282 (m+0) and 285 (m+3) were selectively monitored by GC-MS to determine VLDL-apoB-100 leucine TTR.

Analytical variability

To determine the analytical variability of substrate concentration (plasma FFA, VLDL-TG, and VLDL-apoB-100) and enrichment (palmitate in plasma and VLDL-TG, glycerol in plasma and VLDL-TG, and leucine in plasma and VLDL-apoB-100) measurements, a mock tracer infusion was performed in one man, and the fresh plasma samples were pooled as necessary to obtain sufficient sample volumes for repeated processing (four to five replicates) of five samples. To determine the analytical variability of measuring plasma FFA concentrations and the enrichments of plasma free palmitate, glycerol, and leucine, each plasma sample was frozen immediately after collection ($-80°C$ for at least 7 days), then thawed and processed in quadruplicate as described above. To determine the analytical variability of VLDL-TG and VLDL-apoB-100 concentration measurements, VLDL fractions from each fresh plasma sample were prepared in quintuplicate by ultracentrifugation. VLDL-apoB-100 concentration was measured immediately after collection; the remaining VLDL fractions were frozen ($-80°C$ for at least 7 days), then thawed and assayed for VLDL-TG concentration. To determine the analytical variability of enrichment measurements in VLDL-TG (glycerol and palmitate) and VLDL-apoB-100 (leucine), the five VLDL fractions from each plasma sample were frozen immediately after collection ($-80°C$ for at least 7 days), then thawed and processed as described above. The instrumental (GC-MS) reproducibility for determining TTRs was evaluated by multiple injections of the same processed samples.

Calculations

Palmitate rate of appearance (Ra) in plasma was calculated by dividing the palmitate tracer infusion rate (μmol/min) by the average plasma palmitate TTR value between 60 and 240 min during physiologic and isotopic steady state; total FFA Ra was calculated by dividing palmitate Ra by the proportional contribution of palmitate to total plasma FFA concentration (29).

A metabolic steady state existed with regard to VLDL-TG and VLDL-apoB-100 kinetics because plasma VLDL-TG and VLDL-apoB-100 concentrations remained constant throughout the 12 h sampling period (see Results). The fractional turnover rate (FTR) of VLDL-TG was determined by fitting the TTR time courses of free glycerol in plasma and glycerol in VLDL-TG to a compartmental model (**Fig. 1**) (7, 13, 28, 30). The total rate of VLDL-TG secretion (μmol/min), which represents the total amount of VLDL-TG secreted by the liver, was calculated by multiplying the FTR of VLDL-TG (pools/min) by the concentration

Model for VLDL kinetics
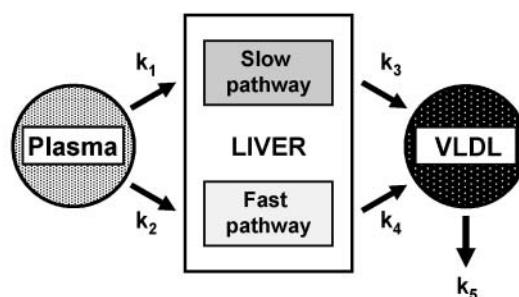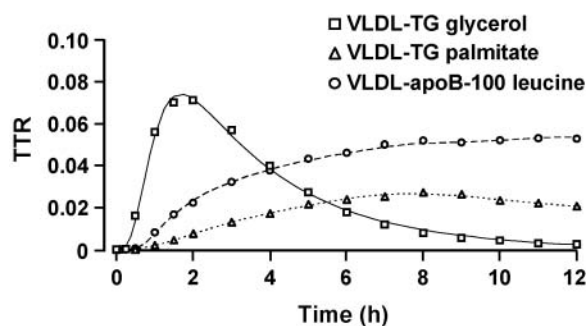
Model fit to experimental data

Fig. 1. The model used for the calculation of VLDL kinetics (top) and a representative model fit to the tracer-to-tracee ratio (TTR) data from one subject for VLDL-triglyceride (TG) glycerol (scaled × 15 for presentation), VLDL-TG palmitate, and VLDL-apolipoprotein B-100 (apoB-100) leucine (bottom). The tracer is introduced into plasma, taken up by the liver, and incorporated into VLDL-TG (glycerol and palmitate) or VLDL-apoB-100 (leucine). Incorporation of tracer into VLDL can occur via two pathways: a fast pathway with only a short time delay until the appearance of label in VLDL (i.e., direct incorporation after uptake from plasma or temporary incorporation into rapidly turning over pools in the liver before incorporation into VLDL), and a slow pathway that accounts for tracer recycling (7, 13, 28, 30).

of VLDL-TG in plasma (μmol/l) and the plasma volume (liters) (10, 11, 13); plasma volume was assumed to equal the VLDL-TG volume of distribution and was calculated as 0.055 l/kg fat-free mass (31). VLDL-TG plasma clearance rate (ml plasma/min) was calculated by dividing the VLDL-TG secretion rate by VLDL-TG concentration in plasma (i.e., FTR × plasma volume). The mean residence time (MRT) of VLDL-TG (min) was calculated as 1/FTR. The MRT indicates the average time that VLDL-TG circulates in the bloodstream; a short VLDL-TG MRT indicates quick removal of TG from circulating VLDL particles, whereas a long MRT suggests that VLDL-TG, after being secreted by the liver, remains in plasma without being hydrolyzed for a considerable amount of time.

The relative contribution of systemic plasma FFA to all fatty acids in VLDL-TG was calculated by the principle of isotopic dilution upon fitting the palmitate TTR in plasma and VLDL-TG to a compartmental model (Fig. 1) (7, 13, 28, 30). These fatty acids represent FFAs from the systemic circulation that are taken up by the liver and directly incorporated into VLDL-TG or temporarily incorporated into rapidly turning over intrahepatic and intraperitoneal TG stores before incorporation into VLDL-TG. The remaining fatty acids in VLDL-TG (nonsystemic) are derived from

pools of fatty acids that are not labeled with tracer during the infusion period; these include *i*) fatty acids released from pre-existing, slowly turning over lipid stores in the liver and tissues draining directly into the portal vein; *ii*) fatty acids resulting from lipolysis of plasma lipoproteins that are taken up by the liver; and *iii*) fatty acids derived from hepatic de novo lipogenesis (32).

The FTR of VLDL-apoB-100 was calculated by fitting the TTR time courses of free leucine in plasma and leucine in VLDL-apoB-100 to a compartmental model (Fig. 1) (7, 13, 28, 30). The rate of VLDL-apoB-100 secretion (nmol/min), the plasma clearance rate of VLDL-apoB-100 (ml plasma/min), and the MRT (min) of VLDL-apoB-100 (indices of the secretion rate, plasma clearance rate, and MRT of VLDL particles) were calculated based on plasma VLDL-apoB-100 concentration and VLDL-apoB-100 FTR as described above for VLDL-TG. The MRT of VLDL-apoB-100 indicates the average time that the VLDL particle remains in the circulation after being secreted by the liver. A molecular mass of 512,723 g/mol for apoB-100 was used for unit conversions (33).

Statistical analysis

All data sets were normally distributed according to the Anderson-Darling normality procedure. The TTR time courses of glycerol and palmitate in VLDL-TG and leucine in VLDL-apoB-100, and the concentrations of VLDL-TG and VLDL-apoB-100 during the 12 h infusion period on the 2 study days, were evaluated using repeated-measures ANOVA. All other results from the two isotope infusion studies were compared with Student's paired, two-tailed *t*-test. Data are presented as means ± SD. $P \leq 0.05$ was considered statistically significant.

Reproducibility statistics (i.e., change in the mean, total error, typical error) and sample size estimations were computed according to Hopkins (34). The change in the mean represents the average difference between the paired individual values obtained in the two studies; it consists of a random change component and a systematic (nonrandom) change component. The 95% confidence interval (CI) of the change in the mean was calculated to assess the absence (when the 95% CI spans 0) or the presence (when the 95% CI does not include 0) of a systematic change. The total error of measurement, or coefficient of variation (CV) reflecting total measurement variability, was calculated as the average of the individual SDs for the values obtained on the 2 different study days divided by the mean of the two values and is expressed as a percentage. Biological variability was estimated from the total measurement variability and the analytical variability as follows: biological CV = square root of (total CV² − analytical CV²) (35, 36). Analytical CV was calculated as the SD of the repeated measurements for each sample divided by their mean. This represents a maximum estimate for analytical variability in our measurements, because analysis of serial samples tends to reduce variability and narrow the 95% CI of analytical determinations (36–38); thus, the analytical CV for the estimation of various kinetic parameters derived from a time series of TTRs and/or concentrations (e.g., plasma palmitate TTR at steady state, VLDL-TG and VLDL-apoB-100 pool sizes, and FTRs) is expected to be equal to or lower than the analytical CV derived from repeated processing and analysis of a single sample. The typical error of measurement was used to estimate the required sample size as a function of the minimum detectable difference for key outcome variables, assuming $\alpha = 0.05$ and $\beta = 0.20$, for both paired and cross-sectional experimental designs. The typical error reflects within-subject variability, includes all sources of error, and was calculated as the SD of the change in the mean divided by the square root of 2. Bland-Altman plots were constructed and examined for the presence of heteroskedasticity, which would indicate that the typical error differs in some systematic way between subjects.

RESULTS

Basal metabolic profile on the 2 study days

Fasting plasma glucose (4.9 ± 0.4 and 4.9 ± 0.3 mmol/l; $P = 0.844$), insulin (21 ± 9 and 20 ± 5 pmol/l; $P = 0.693$), total TG (0.81 ± 0.18 and 0.77 ± 0.20 mmol/l; $P = 0.365$), and total apoB-100 (1.20 ± 0.22 and 1.25 ± 0.17 μmol/l; $P = 0.414$) concentrations were not different on the 2 study days.

Analytical variability of substrate concentration and enrichment measurements

The analytical CV for measuring plasma FFA, VLDL-TG, and VLDL-apoB-100 concentrations was <5%, and the analytical CV for measuring the enrichments of palmitate in plasma and VLDL-TG, glycerol in VLDL-TG, and leucine in plasma and VLDL-apoB-100 was <4.5%; the analytical CV for the determination of glycerol TTR in plasma was slightly higher (<8%) as a result of the very low plasma glycerol enrichments (TTR < 0.015) (**Table 1**). The instrumental variability for enrichment measurements using GC-MS was 0.6% for plasma palmitate, 2.4% for VLDL-TG palmitate, 3.3% for plasma glycerol, 3.6% for VLDL-TG glycerol, 0.9% for plasma leucine, and 2.4% for VLDL-apoB-100 leucine.

Reproducibility of FFA, VLDL-TG, and VLDL-apoB-100 concentrations and kinetics

There were no significant differences in average plasma FFA, VLDL-TG, and VLDL-apoB-100 concentrations between the two studies (**Table 2**). VLDL-TG and VLDL-apoB-100 concentrations remained steady during the 12 h sampling period (**Fig. 2**); there was no effect of time ($P = 0.583$ and 0.776, respectively) or study day ($P = 0.929$ and 0.976, respectively).

The model provided a good fit to VLDL-TG glycerol, VLDL-TG palmitate, and VLDL-apoB-100 leucine TTRs; a representative model fit to the data from one subject is shown in Fig. 1. Average TTR time courses for glycerol and palmitate in VLDL-TG and leucine in VLDL-apoB-100 during the two infusion studies are shown in **Fig. 3**; there were no significant differences between study 1 and study 2 for VLDL-TG glycerol ($P = 0.947$), VLDL-TG palmitate

TABLE 1. Analytical variability

Parameter	Analytical CV
	%
Concentrations	
Plasma palmitate	3.5
Total plasma FFA	3.6
VLDL-TG	4.9
VLDL-apoB-100	3.5
Enrichments	
Plasma free palmitate	1.3
Plasma free glycerol	7.8
Plasma free leucine	1.6
VLDL-TG palmitate	4.0
VLDL-TG glycerol	4.0
VLDL-apoB-100 leucine	4.3

apoB-100, apolipoprotein B-100; CV, coefficient of variation; TG, triglyceride.

TABLE 2. Reproducibility of plasma FFA, VLDL-TG, and VLDL-apoB-100 concentrations and kinetics assessed on 2 separate study days

Parameter	Unit	Study 1	Study 2	Average	95% Confidence Interval	CV
				Change in the Mean		
						%
FFA concentration	mmol/l	0.30 ± 0.10	0.33 ± 0.16	0.03	−0.06, 0.12	24.1
FFA rate of appearance	μmol/min	207 ± 63	235 ± 79	28	−27, 83	21.1
VLDL-TG concentration	mmol/l	0.47 ± 0.14	0.48 ± 0.15	0.01	−0.09, 0.10	14.4
VLDL-TG fractional turnover rate	pools/h	0.451 ± 0.124	0.456 ± 0.093	0.005	−0.138, 0.148	22.2
Hepatic VLDL-TG secretion rate	μmol/min	11.8 ± 2.3	12.4 ± 2.8	0.6	−2.5, 3.7	15.5
Contribution of systemic plasma FFA to all fatty acids in VLDL-TG	%	77 ± 17	74 ± 12	−3	−17, 10	10.1
VLDL-TG plasma clearance rate	ml/min	26.5 ± 7.8	27.0 ± 6.7	0.5	−8.0, 9.0	22.7
VLDL-TG mean residence time	min	141 ± 35	137 ± 30	−4	−46, 37	22.2
VLDL-apoB-100 concentration	nmol/l	62.3 ± 17.2	62.4 ± 20.5	0.1	−12.6, 12.8	12.7
VLDL-apoB-100 fractional turnover rate	pools/h	0.342 ± 0.107	0.353 ± 0.143	0.011	−0.121, 0.143	24.3
Hepatic VLDL-apoB-100 secretion rate	nmol/min	1.19 ± 0.32	1.20 ± 0.43	0.01	−0.24, 0.27	15.3
VLDL-apoB-100 plasma clearance rate	ml/min	19.9 ± 5.7	20.9 ± 8.8	1.0	−6.5, 8.5	23.9
VLDL-apoB-100 mean residence time	min	195 ± 76	211 ± 139	16	−63, 94	24.3
Molar ratio of VLDL-TG and VLDL-apoB-100 secretion rates		10,408 ± 2,569	11,815 ± 6,363	1,407	−3,335, 6,148	18.5

Values are means ± SD.

(P = 0.473), and VLDL-apoB-100 leucine (P = 0.781) TTRs. Consequently, there were no significant differences in average FFA Ra, VLDL-TG and VLDL-apoB-100 FTRs, hepatic VLDL-TG and VLDL-apoB-100 secretion rates, the molar ratio of VLDL-TG and VLDL-apoB-100 secretion rates (an index of the average TG content of the newly secreted VLDL particles), and VLDL-TG and VLDL-apoB-100 plasma clearance rates and MRTs between the two studies (Table 2). In all cases, the 95% CI for the change in the mean included 0, indicating the absence of systematic changes (Table 2). Overall, the intraindividual day-to-day variability (i.e., CV) ranged from ∼15% to ∼25% (Table 2);

Fig. 2. VLDL-TG (top) and VLDL-apoB-100 (bottom) concentrations during the 12 h infusion on the 2 study days. Values are means ± SD.

given our analytical CVs, almost all (>92%) of the total measurement variability is of biological origin. Bland-Altman plots for the typical error (data not shown) revealed no signs of heteroskedasticity.

Sample size estimations for minimum detectable differences

Minimum detectable differences for key outcome variables as a function of sample size in a paired study design, assuming α = 0.05 and β = 0.20, are shown in **Fig. 4**. For cross-sectional studies, the required total number of subjects can be obtained by multiplying the number required for a paired design by 4. For example, if six subjects are required in a paired design, a total of 24 subjects (12 for each group) are required in a cross-sectional design with two groups. The most robust outcome variable was hepatic VLDL-apoB-100 secretion rate, followed by FFA Ra and hepatic VLDL-TG secretion rate (Fig. 4). We found that only six subjects are required with a paired study design to detect a ≥25% difference in FFA Ra, VLDL-TG, and VLDL-apoB-100 secretion rates. To detect differences of the same magnitude in plasma clearance rates and MRTs with a paired study design, 8–9 subjects are needed for VLDL-TG and 13–14 subjects are needed for VLDL-apoB-100 (Fig. 4).
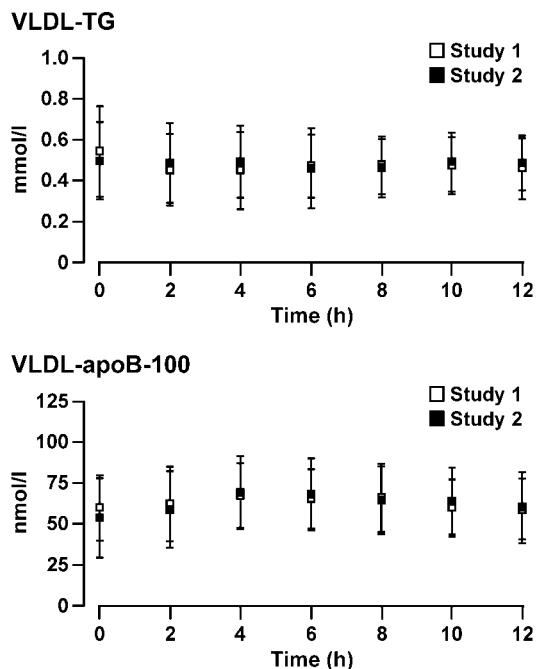
DISCUSSION

In this study, we examined the reproducibility of basal plasma FFA, VLDL-TG, and VLDL-apoB-100 kinetics in healthy male volunteers. Our results indicate that intraindividual day-to-day variability ranges from ∼15–20% for some of the most robust measurements (e.g., FFA Ra, hepatic VLDL-TG and VLDL-apoB-100 secretion rates) to ∼20–25% for others (e.g., VLDL-TG and VLDL-apoB-100 clearance rates and MRTs). Therefore, physiologically meaningful differences in mean values (i.e., ≥25–30% in mag-

VLDL-TG glycerol

VLDL-TG palmitate
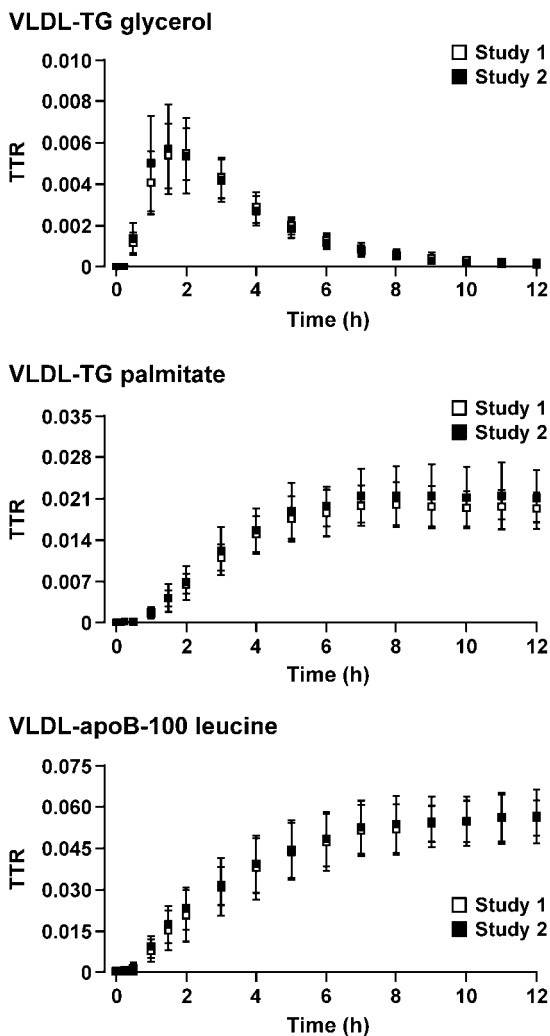

VLDL-apoB-100 leucine

Fig. 3. Average TTR time courses for VLDL-TG glycerol (top), VLDL-TG palmitate (middle), and VLDL-apoB-100 leucine (bottom) during the 12 h infusion on the 2 study days. Values are means ± SD.

nitude) can be obtained with a sample size of 6–10 subjects for paired studies and 12–20 subjects per group for cross-sectional studies, assuming a type I error rate of 0.05 and a type II error rate of 0.20 (i.e., 80% power).
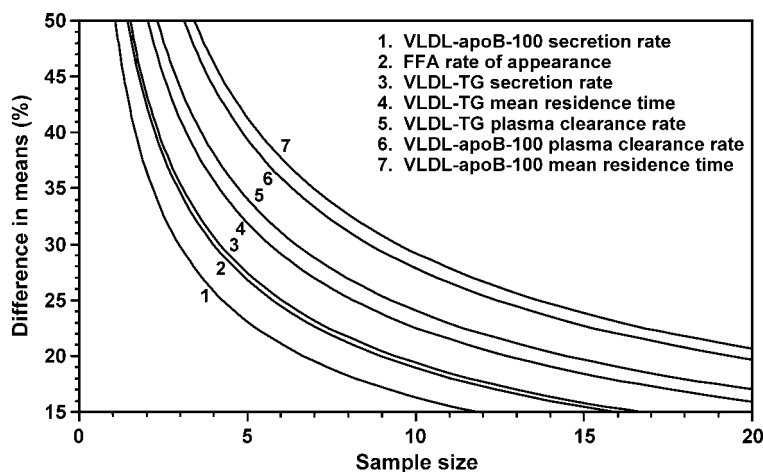
There are no previous studies examining the reproducibility of VLDL-TG kinetics. However, the high day-to-day variability (i.e., 25–30%) of plasma TG concentration measurements often reported in the literature (35, 36, 38–42) could be problematic when evaluating VLDL-TG kinetics. For example, Jacobs and Barrett-Connor (38) investigated the test-retest reliability of fasting plasma TG concentrations in >7,000 men; the 95% CI for a single plasma TG measurement with a true mean of 175 mg/dl was reported to range from 88 to 262 mg/dl. Similarly, Brenner and Heiss (37) evaluated the intraindividual variability in fasting plasma TG concentrations in >2,000 men and reported that the 95% CI for a single plasma TG measurement with a true mean of 150 mg/dl ranges from 93 to 243 mg/dl. In our study, we found that total plasma TG and VLDL-TG concentration measurements varied by only ∼10–15%. This is probably attributable to the more carefully controlled experimental conditions in our metabolic ward study as opposed to epidemiological and clinical settings, because there is evidence that physical activity (43–45) and dietary intake (46, 47) on the day(s) before blood sampling can have significant effects on plasma TG concentration. In another recent investigation, which carefully monitored prestudy dietary intake and physical activity, the variability of plasma TG concentration measurements was indeed as good as that observed in our study (48).

There is one study in the literature that assessed the reproducibility of measuring VLDL-apoB-100 turnover (49). It was reported that the average intraindividual CV (reflecting the total error of measurement) for VLDL-apoB-100 FTR was 23% when six healthy, premenopausal women were studied on the same day of the menstrual cycle during two consecutive cycles. This is very similar to the error estimates for VLDL-apoB-100 FTR and all other outcome variables in the present study in men. In addition, the day-to-day variability reported here in men is no less than the intraindividual variability for basal FFA, VLDL-TG, and VLDL-apoB-100 kinetics that we recently reported when studying premenopausal women once during the follicular phase and once during the luteal phase of the menstrual cycle (11). This finding strengthens our conclusion from the previous study that menstrual cycle

Fig. 4. Minimum detectable differences in plasma FFA, VLDL-TG, and VLDL-apoB-100 kinetics as a function of sample size, assuming $\alpha = 0.05$ and $\beta = 0.20$, in a paired study design. The total sample size (experimental plus control group) required for a cross-sectional study design can be derived by multiplying the number of subjects required for a paired design by 4 (see text for details).

phase does not affect measures of basal plasma FFA, VLDL-TG, and VLDL-apoB-100 kinetics. Moreover, it indicates that the intraindividual day-to-day variability for lipid kinetics is not different in men and women. Therefore, factors that are common to both sexes must be predominantly responsible for day-to-day variations in these measures.

We found that the most robust measurements in our study were those of plasma FFA Ra and hepatic VLDL-TG and VLDL-apoB-100 secretion rates, whereas the assessment of VLDL-TG and VLDL-apoB-100 plasma clearance rates and MRTs was almost twice as variable. This suggests that the factors involved in the control of hepatic VLDL-TG and VLDL-apoB-100 secretion are less variable than those involved in the regulation of intravascular metabolism and the removal of VLDL-TG and VLDL-apoB-100 from the circulation. Based on the reliability of basal plasma FFA, VLDL-TG, and VLDL-apoB-100 kinetic measurements, we calculated the sample size required to detect differences in the means of these outcome variables at the 95% level of significance with 80% power. Clearly, the detection of differences in FFA, VLDL-TG, or VLDL-apoB-100 kinetics that are ≤10% requires a prohibitively large number of study subjects, bearing in mind the cost of tracers and the analytical burden involved. Nevertheless, physiologically meaningful differences (≥25–30%) in basal FFA, VLDL-TG, and VLDL-apoB-100 kinetics can be detected with adequate power (β = 0.20) and a type I error rate of 0.05 with only 6–10 subjects in a paired study design or 12–20 subjects per group in a cross-sectional study design.

In summary, we found that intraindividual differences in tracer measures of plasma lipid kinetics range from 15% to 25%. This is comparable to the intraindividual variability of most biological measurements. Based on these findings, we provided estimates of sample size requirements for studies examining FFA, VLDL-TG, and VLDL-apoB-100 kinetics. This information will be useful for future studies investigating FFA, VLDL-TG, and/or VLDL-apoB-100 kinetics in response to various interventions or in different study populations.⬛

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