REVIEW

Getting the label in: practical research strategies for tracing dietary fat

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The observation that events occurring after consumption of a meal can directly affect metabolic risk has been gaining interest over the past 40 years. As a result, the desire for investigators to conduct postprandial studies has also increased. Study design decisions pertaining to the choice of meal quantity and composition are more difficult than may be readily apparent, and there is now ample evidence available in the literature to suggest that what is fed on the test day significantly affects postprandial metabolism and can therefore influence interpretation of results. In addition, events occurring before the testing day (food intake and activities) can also have an impact on the observed postprandial response. The goal of this review is to present aspects of study design critical to the investigation of postprandial metabolism. These details include subject preparation, meal quantity, form and composition, as well as sampling protocols for measuring metabolites. Key factors and practical examples are provided to minimize the impact of nonresearch variables on subject variability. Finally, aspects related to using stable isotope tracers to measure metabolism of meal fat are discussed, including choice of tracer form, dose and delivery in food. Given that fed-state events contribute significantly to chronic disease risk, improved methods to study the absorption and disposal of food energy will support the development of strategies designed to prevent and treat diseases associated with overconsumption of nutrients.

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INTRODUCTION

The atherogenicity of the postprandial state, first proposed by Donald Zilversmit,¹ has remained the focus of intense research for over 4 decades. A central idea evolving from Dr Zilversmit's hypothesis is that the physiologic response to a meal high in fat reveals key dynamics related to prediction of heart disease risk.² The concept of a nutrient challenge as a vehicle to uncover disease risk has grown to include studies of metabolic flexibility³ and the role of nutrient toxicity during overconsumption of food.⁴ Over the past 20 years, advanced techniques for the study of postprandial lipemia have been developed.^{5–9} Reviews on postprandial triacylglycerols (TG) response have described the influence of genetic polymorphisms,² the impact of subject characteristics such as obesity¹⁰ and the effect of commonly prescribed medications.¹¹ Further, recent research has made major progress in our understanding of the metabolic fates of dietary fatty acids in humans¹² and the large variability exhibited between study subjects with respect to fatty acid handling.¹³ At the same time, much new information has become available with which to optimize postprandial study designs, and the objective of this review is to highlight that information.

It may seem that the impact of detailed study design features, such as feeding liquid vs solid meals, would be so obvious that they would not need elaboration. However, literature demonstrating the importance of the study meal design is somewhat underappreciated. Both the fasting and postprandial concentrations of TG vary more than other metabolites,¹⁴ and as a result of subject variability many studies fail to show significant differences between treatments—mostly owing to lack of power. However, if

the factors put forth here are controlled for, variability in many outcomes will be significantly reduced and hypotheses may be tested with smaller sample sizes and lower costs. It should be noted that the details mentioned below are for recommended study conditions. Obviously, it is to be expected that some study designs and populations, as well as financial and other limitations, will not permit this level of control. However, as so much of the human response to eating cannot be controlled for, every attempt should be made to regulate study design features that can be controlled in practice. The planning of postprandial research has been elegantly described previously,¹⁵ and will be expanded here to include tracer methodology considerations. The review will begin with a focus on strategies needed to control for the prestudy activities of research subjects, followed by a discussion of the composition and energy content of test meals. We conclude with consideration of the types/doses of isotopes used to label meal lipid and the impact of blood sampling frequency and analysis. For the reader's interest, an Appendix is provided that contains details of 20 postprandial studies chosen from the literature to illustrate practical examples of how researchers have designed their studies to solve many of the challenges discussed herein.

RESEARCH SUBJECT PREPARATION

Prestudy activities

Standardization of the research subject's pre-testing activities provides one of the most efficient means to reduce variability in the response to a meal test. In particular, participants should avoid

consuming alcohol 2–3 days before the testing period.¹⁶ In addition, vigorous exercise should be limited before and on the testing day, as it can increase fat oxidation^{17,18} and reduce postprandial lipemia by 20% or more.¹⁹ Even lack of sufficient sleep,²⁰ or walking to the test site, can impact post-meal lipemia²¹ and should be considered when planning research. One of the most significant factors is the content of the meal consumed the evening before the test meal.²² Pre-test food consumption should be controlled or monitored in the days leading up to the testing day. Ideally, prestudy food intake will be provided directly to the subjects by research personnel. However, this is not always possible because of limitations in subject accessibility, cost or other study design restrictions (for example, surgery patients). At the minimum, food intake should be monitored using food records in the days leading up to the testing period, and subjects should be counseled on how to complete these records or be provided scales for measuring food items. Alternatively, some investigators have instructed subjects to either consume or avoid certain foods before the test; for example, avoiding foods naturally enriched in ¹³C, such as corn products and cane sugar, if using ¹³C-tracer methods with the meal test.⁷ One strategy used with larger sample sizes is to instruct subjects to go to a fast-food restaurant and choose from a list of prescreened items on the menu. This method is convenient, allows for standardization of the evening meal food intake and yet gives subjects a variety of items to choose from. It is now recognized that a significant portion of the previous evening meal's TG resides in the enterocyte for 4–16 h after the meal.²³ This lipid will enter the blood at the onset of the next meal—a phenomenon termed the 'second meal effect'. This effect can be reduced by subjects consuming a low-fat evening meal the night before the postprandial study, and such a scheme will maximize detection of the test meal entry.

Composition of the test meal relative to the background or long-term research diet

For research that includes only one test meal per subject, the meal can be the same composition for all subjects. It is helpful to have a measure of habitual dietary intake, as this will affect the clearance of lipids postprandially.²⁴ For studies in which a meal challenge is to be administered both before and after a long-term dietary intervention, the composition of the meal becomes a critical component in the testing of the hypothesis. The composition of the prestudy meals can be specific to the study's hypothesis (that is, high in protein content for a study of long-term elevation in daily protein consumption) or different from the long-term diet. Regardless, consideration should be given to differences between long-term research dietary treatments and the test meal composition. For instance, if a low-fat diet is being fed for research purposes over an extended period of time (weeks to months), then a test meal that is very high in fat that is fed before and after the dietary treatment may result in the appearance of a 'poor' study outcome when postprandial lipemia appears worse after acclimation to a low-fat diet. This result is likely as individuals best metabolize a macronutrient mix most similar to that recently eaten.²⁵ Thus, metabolism of the test meal is influenced by both the background diet and the composition of the meal itself. Probably the most comprehensive test of the acute vs long-term effects of dietary fatty acids was performed by Bergeron and Havel,²⁶ who studied two groups of men—one group fed a high-PUFA diet for 4 weeks and the other group fed a highly saturated fatty acid diet for 4 weeks. At the end of the study, each subject underwent two postprandial tests: one meal matched to the fatty acid composition of their chronic research diet and the other meal test reflecting the composition of the opposite diet. In this way, the authors determined how the chronic dietary treatments changed fasting TG concentrations and determined the acute impact of meal fatty acids on chylomicron production.

MEAL DETAILS

Although this discussion emphasizes factors involved in designing studies of postprandial lipid metabolism, the main principles can be applied to the analysis of any macronutrient of interest. Overall, two details of subject selection relate to the choice of meal contents. First, during the research subject screening process, information should be obtained from the subject that relates to his/her habitual food intake and food tolerances (taste, allergies and lactose intolerance) to exclude ineligible subjects. Second, at some point before the meal test, it is helpful to have a small quantity of the meal vehicle (milkshake without added isotopes) available for the subject to taste to determine its palatability and tolerability. For studies involving more than one meal test, this kind of prestudy introduction to the content of the meal will reduce learning effects (the novelty of the food), which may affect metabolism.

Form of the meal

Both liquid²⁷ and solid²⁸ meals have been used to assess fat absorption and postprandial metabolism.²⁹ Whole-food meals have the benefits of producing results that are more generalizable, yet their solid nature will slow gastric emptying and thus affect absorption and blood concentrations of metabolites. Solid-food test meals also necessitate duplicate tray preparation and homogenization before the foods are subjected to proximate analysis.²⁸ Foods with more complicated preparation requirements such as cutting, chopping or baking take longer to prepare, but may represent more typical food intake. By contrast, simple liquid meals (for example, cream added to a liquid meal replacement) allow for better control of the nutrient composition from subject to subject. If foods are included that naturally vary in composition throughout the year (for example, dairy products³⁰), it is advantageous to take an aliquot of the homogenized meal for analysis of its macronutrient content and fatty acid composition. Further, the use of commercially available liquid formulas allows variability in the quantity fed to different subjects based on their daily energy needs, maintenance of constant food composition and standardization of the meal composition across multiple research sites. For these reasons, it is readily apparent why many test meals, particularly those incorporating stable isotopes, are comprised of a liquid delivery vehicle and are complemented by simple solid foods fed in the same meal (see Appendix).

Meal composition and energy content

As the test meal composition is usually designed to emphasize the macronutrient of interest, the balance of other macronutrients in the meal should be considered. Much previous research has focused on carbohydrate and fat metabolism such that test meals have tended to be low in protein. Early lipid tolerance tests were designed to provide a fat dose that would challenge the body's lipid handling systems.³¹ Later, postprandial studies were designed to better mimic normal patterns of eating by including carbohydrate and protein with the fat and feeding solid foods,³² with the addition of isotopes to meals.²⁷ To quantify postprandial lipid metabolism, obviously, fat should be emphasized in the test meal, but the addition of carbohydrate to the meal will allow an insulin response, which can influence enzymes and transport proteins required for lipid clearance. Blood TG concentrations frequently fall slightly when the test includes only sugars.³³ The meal composition will also affect the incretin response, which can alter peripheral metabolism.³⁴ The final composition of the test meal may be a result of foods chosen on the basis of palatability, availability, cost or, if using stable isotopes, foods providing an efficient vehicle for the tracer.

The quantity of food provided on the test day can be either standardized (the same quantity of food provided to each subject) or individualized (calculated on the basis of the individual's

estimated daily energy requirements). Standardization of food quantities for the test meal has the advantage of being easy to prepare across subjects, but is most appropriate when studying homogenous populations (for example, lean healthy young men). When the population of interest is more diverse, it is preferable that food quantity be allocated based on a fraction of each individual's daily energy requirements obtained from food records, indirect calorimetry or using standard calculations such as the Harris-Benedict equation³⁵ with an adjustment for physical activity. The test meal quantity can reflect the size of a normal meal, typically representing 30–40% of daily energy needs, or if fed first thing in the morning can contain more energy. The quantity can depend on how long the subject will be fasting afterward; if the duration is > 4 h, it may be better to provide a greater quantity of food in the test meal (40–60% energy requirements).

Rate of delivery

Liquid meals have been provided as continuous feedings via duodenal administration³⁶ or periodically sipping a beverage,³⁷ although this form of feeding may result in secretion of smaller chylomicron particles.^{36,37} The transition from the fasted to the fed state requires organs such as the liver and adipose tissue to switch from the mode of providing substrates to the body (glucose and fatty acids, respectively) to a mode of storing the newly available substrates provided by meals. Dysregulation of metabolism is characterized by the inability of the body to shut off the release of these endogenous substrates during simultaneous storage of meal components. Thus, inherent in the ability of postprandial tests to uncover dysregulation of metabolism is the need to provide a bolus of incoming nutrients that mimic normal patterns of food consumption. Furthermore, an efficient 'fasting to feeding switch' may be more easily obtained when that single meal is eaten after a 12-h fast as opposed to a second or third meal eaten in a day. However, the response to meal fat consumption is also affected by individual differences in absorption that are beginning to be appreciated.²³ We have documented the very large variability of individual subjects to absorb a test meal,²³ and shown in Figure 1 are more recent examples of the absorption rate of a tracer consumed at lunch, as the first meal of the day

(time 0). This rise in the lunch label (solid bold line) after the meal can be contrasted with the rise in plasma concentration of TG-rich lipoprotein apoB48 (solid gray line), the marker of chylomicron particle concentration. The evening meal consumed the night before also contained an isotope (denoted by the dashed line). At -2 h in each of these figures, the evening meal label can be seen to be decaying in the blood, having peaked in blood concentration and enrichment during the night, about 10 h earlier. For some subjects (A and B in Figure 1, dashed lines), a distinct peak signifying resecretion of stored intestinal lipid can be detected early in the postprandial period, and the peak is coincident with the increase of apoB48 in TG-rich lipoproteins between 1 and 2 h after lunch. For subjects A and B, the lunch meal isotope is then observed to enter the pool starting at 1 h, and its peak occurs at the same time as a second apoB48 peak. For subject C, between 0 and 1 h, the evening meal label peak is much smaller and the rise in apoB48 tracks more closely with entry of the lunch meal label. By contrast for subject D, the first peak of apoB48 is associated with an almost imperceptible evening meal peak in the TG-rich lipoproteins (~1 h), and the lunch meal label entry does not become apparent until the very end of the 6-h study. The reader can appreciate how the postprandial blood TG concentration among these subjects will be affected by the rate of apoB48

Figure 1. Individual variation in entry of meal fat by comparison of meal stable isotopes in TRL-TG with TRL-apoB48 concentration. These data from four subjects (A–D) show lack of congruence between apoB48 lipoprotein dynamics and isotopic entry of meal fat TG. Subjects were fed a meal at 1800 hours (evening meal) containing a fatty acid isotope and then fasted until noon the next day (18 h), at which time they were fed a second meal (lunch) that contained a different isotope of fat. The graphs present B48 and the sources of TG fatty acids relative to the start of lunch (time 0). The compositions of the meals were identical (40 g fat, 40% of the subject's daily energy), but they contained different isotopes of palmitate homogenized in the chocolate shake. The y axis on the left displays the concentrations of apoB48 in the TRL fraction (denoted by the gray line on the graph). The y axes on the right display the percentages of TRL-TG palmitate as measured by GC/MS. TRL-TG palmitate data from the evening meal are denoted by the dashed black line, whereas palmitate data from the lunch are represented by the solid black line. An example of the interpretation of these data from subject A is as follows: (1) recirculation and decay of evening meal label in the TRL-TG pool 16 h after consumption of the label the night before, such that at this time point ~2.7% of plasma TRL-TG was derived from the previous evening meal. (2) Rise in apoB48 concentration 15–30 min within the onset of eating. (3) Resurgence of evening meal label in TRL-TG coincident with secretion of lunch apoB48-containing particles. (4) Entry of lunch meal label. (5) Second smaller peak of evening meal label coincident with continued evidence of lunch label in TRL-TG. (6) Second peak of B48 coincident with peak of lunch TG label absorption.

particle secretion, the capacity to store intestinal lipid and the mix of lipid pools being used for chylomicron-TG synthesis. Future research is needed to understand these significant differences in absorption of meal fatty acids.

Sampling protocol

The convention of sampling post-meal blood at multiple time points increases the costs of these studies. Figure 2 depicts the relative postprandial time courses of common analytes after consumption of solid high-carbohydrate, high-fat or mixed meals, designed to give the reader an idea of time to peak concentrations and clearance profiles. Consumption of liquid meals will affect these curves, resulting in faster entry and clearance of metabolites. The graphs depicted are theoretical time courses expected in normal healthy humans; patients with disease features affecting postprandial metabolism (for example, diabetes, hyperlipidemia) will often have postprandial metabolic curves that are amplified and extended, and these considerations should be incorporated into the study design (that is, the sampling period should be extended). The frequency and length of sampling intervals will depend on the outcome of interest. For example, if meal fat incorporation into chylomicron-TG is the primary aim, sampling should occur early in the postprandial period (0.5 h) and extend to 4 h or more, whereas meal fat incorporation into very low-density lipoprotein (VLDL)-TG is slower (Figure 2). Glucose and insulin, on the other hand, with their rapid time courses, peak at ~60 min and return to baseline within 120 min, and therefore require more rapid sampling early in the postprandial period.²⁷

TRACER CONSIDERATIONS

Historically, postprandial fat metabolism was investigated by labeling TG-rich lipoproteins with retinyl palmitate and measuring the disappearance of retinyl palmitate from the plasma (although this method may have limited utility in the late postprandial period).³⁸ Technical advancements have included an increased availability of stable isotopes and technologies designed to analyze isotopically labeled compounds using gas chromatography coupled with either mass spectrometry or isotope ratio mass spectrometry. The higher sensitivity of isotope ratio mass spectrometry allows for the detection of lower enrichment; therefore, smaller doses of tracer can be used. There are many excellent reviews on the theory and principles underlying tracer methodology that can be used for reference.^{39,40}

Form of isotope used

The choices of dietary isotopes can include fatty acids fed in the free form (for example, ¹³C₄-palmitate) or as TG (d₃₁-tripalmitin). For the purposes of studying metabolism of dietary fat, either of these forms can be used. However, free fatty acids are rarely consumed by humans, as the bulk of fatty acids in foods are present in TG form. In fact, free fatty acids are disliked by humans,³⁴ and if the isotope is synthesized in the form of a salt (sodium and potassium), this can worsen the taste making it bitter and soapy. The chemical form of the tracer strongly influences its solubility.⁴¹ If the fatty acid salt is solubilized in water, it must be handled gently or it will create a foam and result in a food product more difficult to prepare and ingest (Lambert/Parks, personal observations). Incorporation of the fatty acid isotope into food has typically been achieved by emulsifying it into a sugar-protein complex or vegetable oil, and mixing it into food or a milkshake-type beverage. In addition, the vehicle for the tracer often must be heated, sonicated or emulsified using a homogenizer readily available in food processing labs³⁶ to efficiently incorporate the tracer. TG isotopes are more easily incorporated into test meals if they are in the form of an oil, such as triolein; whereas if the tracer is a crystal, such as tripalmitin, it must be heated while being

Figure 2. Theoretical time course curves representing typical postprandial metabolism of common analytes, including plasma glucose (a), insulin (b), and NEFA (c), and TG from plasma (d), CM (e), and VLDL (f), after consumption of a glucose-only meal challenge, a high-fat meal challenge or a meal of mixed macronutrient composition. The figures are presented to aid in the development of postprandial study designs, to portray the differing time courses of metabolites after meals of varying macronutrient composition. These curves represent typical concentration changes after consumption of single meals in healthy human subjects. The y axes represent concentration changes relative to baseline. Typical macronutrients might be (1) glucose-only (solid black line with circles): 100% of energy from CHO; (2) high-fat (solid black line): 28% CHO, 60% fat, 12% protein; or (3) mixed composition (dashed black line): 55% CHO, 30% fat, 15% protein. Consumption of a second meal would induce secondary peaks. In the case of TG metabolism, secondary peaks will frequently be larger than the first, and will naturally delay the return of TG concentrations to baseline. CHO, carbohydrate; CM, chylomicron; NEFA, nonesterified fatty acids; TG, triacylglycerol; VLDL, very low-density lipoprotein.

mixed into the vehicle (for example, butter) or solubilized using the right combination of fats (oils, butter) and emulsifiers (free cholesterol or pasteurized egg yolk). Higher amounts of isotope, even in TG form, are increasingly difficult to incorporate effectively into a carrier vehicle by conventional methods. For example, large amounts of tripalmitin (3–4 g) solubilized in heated oil will begin to solidify if mixed with cold foods. The recrystallization of the isotope in the milkshake will reduce its absorption. Therefore, consideration and experimentation is necessary to ensure that the tracer is effectively incorporated into the vehicle.

Quantity of isotope and the food vehicle

The dose of the isotope will depend on the solubility and palatability of the compound, the enrichment desired and the

dietary fat content of the meal (Appendix). Isotopes incorporated into large endogenous pools that turn over slowly (for example, dietary fat moving into adipose TG) will need to be administered in higher amounts and for longer periods of time compared with isotopes that are incorporated into pools that are smaller and turn over more rapidly (plasma TG). For example, when measuring fatty acid oxidation by analyzing labeled carbons in CO_2 , U^{13}C -palmitate may be preferable to $^{13}\text{C}_4$ -palmitate, as all carbons in U^{13}C -palmitate have the potential to label CO_2 . Further, some investigators may choose to use a greater amount of meal isotope to obtain more accurate assessment of enrichments in plasma at earlier postprandial time points, when the label is just entering the body. Technically, quantities of isotope that result in a 10–20% enrichment of the chylomicron pool would not be referred to as 'tracers'.^{27,28} Conversely, the ratio of the tracer (for example, 100–800 mg) to the amount of total dietary fat (35–40 g) may be much lower (<3%) depending on the limits of the detection method (Appendix). Increasing the amount of dietary fat in the meal leads to higher postprandial plasma-TG concentrations⁴² that can dilute the appearance of the label, but also amplify early plasma lipid concentrations to a level sufficient for effective mass spectrometry analysis. A greater dose of label may be required in hypertriglyceridemic subjects, because of the dilution of the label with greater nonlabeled TG in plasma. If a large dose of isotope is given (for example, 1–2 g), it is likely that an adequate enrichment in the plasma pools will be achieved regardless of individual differences in plasma TG concentrations.

Capsules have been used to deliver tracer lipids,^{23,43} but one study reported that the recovery of ^{13}C in breath was 10 times higher when the tracer was fed in the meal than in capsule form.⁴⁴ Many investigators have fed only a single isotope-containing meal and therefore measure the acute postprandial response to that meal. However, typical meal consumption in free-living subjects means that plasma TG concentrations progressively rise over the course of the day.⁴⁵ The bulk of this change in TG is a result of increases in chylomicron-TG and VLDL-TG concentrations (Figure 2). This rise reflects 'back-up' of clearance in TG-rich lipoproteins, which increases steadily, peaking at night at about 0200 hours, after which time plasma-TG concentrations fall steadily until morning. As demonstrated in Figure 1, for some subjects who absorb meal fat slowly, a second meal may be required to see maximum appearance of the dietary tracer in plasma lipid compartments.^{23,46} After two identically labeled meals, the enrichment of chylomicron-TG pool can approach 100% that of the meal.²⁷ To maximize entry of a label given in a fat-containing breakfast meal, it is not necessary that fat be included in the second meal; rather, the secondary release of chylomicron-TG may be stimulated by providing a meal containing only glucose.⁴⁷

Administration and sampling considerations

Controlling three details associated with meal consumption can improve the measurements of dietary fat absorption. The first detail is to standardize the time associated with complete consumption of the meal by the subjects (10–20 min for most meals). Consumption of the meal in a restricted time frame will limit differences in gastric emptying between subjects. Second, various compounds can be consumed with the meal to give estimates of gastric emptying. For example, acetaminophen⁴⁸ is water soluble and not absorbed in the stomach, and therefore its appearance in plasma gives an estimate of intestinal absorption. Finally, to ensure that the meal containing the isotope is completely consumed, investigators typically rinse the plate, cup or bowl carrying the meal with water for the subject to consume. Before and after food consumption, the frequency and duration of blood sampling intervals will depend on the outcome of interest. Before the meal begins, it is advisable to obtain blood at a

minimum of two time points for the measurement of plasma TG and average these values to represent the fasted concentrations.¹⁴ After meal consumption, if isotope incorporation into chylomicron-TG is the primary aim, sampling should occur often (every 20 min) early in the postprandial period (0–3 h) and extend to 4 h or more. Incorporation of the meal isotope into VLDL begins around 3–4 h after the onset of the meal and continues to increase thereafter.^{27,49}

CONCLUSION

Advances in the measurement of postprandial metabolism have supported a better understanding of the health consequences of single meal components, foods and eating patterns. Given the challenges of studying metabolism acutely after meals (that is, in the non-steady state) and the high variability in blood TG concentrations, specific study design constraints can be adopted during the research planning stage to increase the likelihood of successful hypothesis testing. These constraints include documenting the subjects' habitual food and activity patterns and controlling their exercise, food and alcohol intakes in the days before the postprandial test. With regard to the composition of test meals, trends toward feeding mixed meals, of moderate fat content (40–50 g), have increased the generalizability of research findings. Stable isotopes have been added to these meals, which have been made up of liquid formulas (convenient), solid foods (more physiologic) and a combination of these two vehicles. Milkshakes and puddings have been used as isotope-delivery vehicles, and isotopes have been baked directly into the food. Overall, the success of using isotopes to track meal fat absorption/metabolism strongly depends on ensuring that the label is homogeneously solubilized in the food. Isotope methodology and the kinetic measurements that can be made using this methodology stand at the center of trans-disciplinary research. The hope of this review is to facilitate the integration of postprandial work into multidisciplinary efforts toward the goal of testing the effects of social and behavioral factors (effects of stress, eating situation and exercise) on metabolic risk and the development of obesity-associated chronic diseases.

CONFLICT OF INTEREST

EJP has received consulting fees from Atkins Nutritionals, Inc. and Merck. JEL declares no conflict of interest.

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APPENDIX

Features of study design and tracer methodology impacting postprandial lipid metabolism

Author, reference study population	Tracer and dose	Tracer vehicle	Test meal	Pre-study meal details
Jones <i>et al.</i> ⁴³ Healthy men (n = 4)	20 mg kg ⁻¹ of ¹³ C ₁ -stearic acid 10 mg kg ⁻¹ of ¹³ C ₁ -oleic acid 10 mg kg ⁻¹ of ¹³ C ₁ -linolenic acid	Capsules	Composition: 40% fat, 47% CHO, 13% protein Quantity: 1/3 daily energy requirements Second meal: yes (3 isocaloric meals over the day)	Length: 6 days Composition: 40% fat, 47% CHO, 13% protein Quantity: calculated energy requirement (not described)
Murphy <i>et al.</i> ⁵⁰ Healthy women (n = 6)	10 mg kg ⁻¹ of ¹³ C ₁ -palmitic acid	Butter (10 g; in meal)	Composition: 120 g bread, 100 g orange juice, 10 g butter Quantity: 1667 kJ Second meal: no	Length: 2 days Composition: self-selected; avoided naturally ¹³ C-enriched foods Quantity: self-selected
Emken <i>et al.</i> ⁵¹ Healthy men (n = 6)	3.5 g of d ₂ -linoleic acid	100 g non-fat yogurt	Composition: 30% fat, 55% CHO, 15% protein Quantity: not described Second meal: yes	Length: 15 days and throughout crossovers Composition: 30% fat, 55% CHO, 15% protein Quantity: estimated energy requirement
DeLany <i>et al.</i> ⁴⁴ Healthy men (n = 4)	10 mg kg ⁻¹ each of ¹³ C ₁ -laurate, palmitate, stearate, oleate, elaidate, linoleate, linolenate	Ensure [®] ; heated	Composition: liquid meal replacement Quantity: not described Second meal: yes; same as breakfast	Length: 7 days Composition: 40% fat, 45% CHO, 15% protein Quantity: estimated energy requirement (not described)
Gill <i>et al.</i> ¹⁸ Healthy men (n = 11)	10 mg kg ⁻¹ of ¹³ C ₁ -tripalmitin	Heated emulsion with lipid, casein, glucose, sucrose	Composition: 99 ± 18 g fat, 102 ± 18 g CHO, 23 ± 4 g protein (cream, oats, fruit, chocolate, nuts) Quantity: 5.7 ± 1.0 MJ; calculation not described Second meal: yes (given 8 h later)	Length: 2 days Composition: self-selected; avoided naturally ¹³ C-enriched foods Quantity: self-selected; monitored by food records
Votruba <i>et al.</i> ⁵² Healthy (n = 9)	2 mg kg ⁻¹ of ¹³ C ₁ -acetate 10 or 15 mg kg ⁻¹ of ¹³ C ₁ -palmitic acid	Boost [®] ; heated	Composition: 35% fat, 50% CHO, 15% protein Quantity: 20% estimated (BEE × 1.5) Second meal: yes	Length: none Composition: NA Quantity: NA
Evans <i>et al.</i> ⁵³ Healthy (n = 8)	800 mg of ¹³ C ₁ -palmitate 800 mg of ¹³ C ₁ -oleate	Emulsified into chocolate milkshake	Composition: 37 g fat, 100 g CHO, 16 g protein (cornflakes, whole milk, chocolate, banana, and shake containing tracer) Quantity: ~800 kcal Second meal: no	Length: evening before Composition: self-selected but low fat Quantity: self-selected
Burdge <i>et al.</i> ⁵⁴ Healthy women (n = 6)	700 mg of U ¹³ C-linolenic acid	Shake ^a ; heated	Composition: 41% fat, 49% CHO, 10% protein Quantity: 3995 kJ Second meal: yes	Length: 1 day Composition: not described Quantity: 11 MJ per day
Punyadeera <i>et al.</i> ⁵⁵ Lean and obese white and black women (n = 8–10 each group)	10 mg kg ⁻¹ of ¹³ C ₁ -palmitic acid	Emulsion ^b	No other foods provided besides tracer emulsion Second meal: no	Length: none Composition: NA Quantity: NA
Pawlosky <i>et al.</i> ⁵⁶ Healthy (n = 10)	1 g of d ₅ -linolenic acid	Low-fat yogurt	Composition: not described Quantity: standardized but not described Second meal: yes	Length: 3 weeks Composition: 33% fat, 51% CHO, 16% protein Quantity: estimated energy requirements via Harris–Benedict equation

APPENDIX (Continued)

Author, reference study population	Tracer and dose	Tracer vehicle	Test meal	Pre-study meal details
Donnelly <i>et al.</i> ²⁸ NAFLD patients (<i>n</i> = 9)	1–2 g of d ₃₁ -tripalmitin (10% enrichment of diet)	Whole foods (details NA)	Composition: 30% fat, 55% CHO, 15% protein Quantity: not described Second meal: no	Length: 3 days Composition: 30% fat, 55% CHO, 15% protein Quantity: estimated energy requirements via Harris–Benedict equation with activity factor adjustment
Timlin <i>et al.</i> ²⁷ Healthy (<i>n</i> = 6)	1.6 g (average) of d ₃₁ -trihexadecanoate	Shake (Boost [®] , egg yolk, cream, vegetable oil); heated	Composition: 32% fat, 54% CHO, 14% protein Quantity: 2/3 daily energy requirements via Harris–Benedict equation Second meal: yes	Length: 3 days Composition: 30% fat, 55% CHO, 15% protein Quantity: estimated energy requirements via Harris–Benedict equation
Votruba <i>et al.</i> ⁵⁷ Healthy women (<i>n</i> = 6)	10 mg kg ^{−1} of ¹³ C ₁ -oleic acid 15 mg kg ^{−1} of d ₃₁ -palmitic acid	Boost [®] ; heated	Composition: 30% fat, 55% CHO, 15% protein Quantity: 30% daily energy requirement estimated by (BEE × 1.4) Second meal: yes	Length: 1 day Composition: 30% fat, 55% CHO, 15% protein Quantity: estimated energy requirements using (BEE × 1.8)
Barrows and Parks ⁵⁸ Healthy men (<i>n</i> = 12)	d ₃₁ -tripalmitin (20% enrichment)	Shake (Boost [®] , cream, oil); heated	Composition: 32% fat, 54% CHO, 14% protein Quantity: 2/3 daily energy Second meal: no, continuous feeding	Length: 3 days Composition: 30% fat, 55% CHO, 15% protein Quantity: estimated energy requirements via Harris–Benedict equation with activity factor
Turpeinen <i>et al.</i> ⁵⁹ Healthy (<i>n</i> = 15)	50 mg of U ¹³ C-linoleic acid 50 mg of U ¹³ C-linolenic acid	Sugar cube	Composition: not described; provided muffin (containing 10 g olive oil), orange juice, fat-free yogurt Quantity: not described Second meal: yes	Length: 14 days. Composition: not described Quantity: some meals standardized; specific ingredients provided to subjects
Bickerton <i>et al.</i> ⁶⁰ Healthy (<i>n</i> = 14)	100 mg of U ¹³ C-palmitate	Olive oil emulsion	Composition: 40 g fat, 40 g CHO (olive oil emulsion and cereal with skim milk) Quantity: as above Second meal: no	Length: none Composition: self-selected; avoided naturally ¹³ C-enriched foods for 48 h Quantity: self-selected
Heath <i>et al.</i> ⁶¹ Healthy men (<i>n</i> = 8)	600 mg of ¹³ C ₁ - palmitate (breakfast) 450 mg of ¹³ C ₁ -oleate (lunch)	Test oil or milkshake	Composition: 51.9 g fat, 76.4 g CHO, 12.2 g protein Quantity: as above Second meal: yes	Length: evening before Composition: self-selected but low fat Quantity: self-selected
Hodson <i>et al.</i> ⁶² Healthy (<i>n</i> = 12)	100 mg of U ¹³ C-oleate 100 mg of U ¹³ C-palmitate 100 mg of U ¹³ C-linolenic acid	Chocolate–oil emulsion; heated	Composition: 40% fat, 45% CHO, 15% protein Quantity: 1/3 energy requirements estimated by Schofield equation Second meal: yes	Length: none Composition: self-selected; avoided naturally ¹³ C-enriched foods for 48 h Quantity: self-selected
Ruge <i>et al.</i> ⁶³ Healthy (<i>n</i> = 8)	100 mg of U ¹³ C-linolenic acid (breakfast) 100 mg of U ¹³ C-oleate (lunch) 100 mg of U ¹³ C-palmitate (dinner)	Chocolate–oil emulsion; heated	Composition: 39% fat, 53% CHO, 15% protein Quantity: 1/3 energy requirements estimated by (BEE × 1.25) Second meal: yes	Length: none Composition: self-selected Quantity: self-selected
Chavez-Jauregui <i>et al.</i> ⁶⁴ Healthy (<i>n</i> = 10)	¹³ C ₂ -triolein (evening meal) ¹³ C ₇ -triolein (breakfast)	Shake (milk, olive oil, chocolate syrup) Capsules	Composition: 30 g fat (capsules); meal 33 g fat, 124 g CHO. Quantity: as above Second meal: yes	Length: 3 days Composition: self-selected for 3 days; standardized evening meal containing tracer Quantity: estimated energy requirements using Harris–Benedict equation

Abbreviations: BEE, basal energy expenditure; CHO, carbohydrate; NA, not available; NAFLD, non-alcoholic fatty liver disease.
Second meal indicates whether a second meal was consumed; here assumed to be a meal occurring within 4 h after the initial test meal (unless otherwise noted).

^aShake: 22 g cream, 12 g casein, 4.5 g beet sugar, 9 g sugar, 10 g chocolate powder; oil.

^bEmulsion: 22 g cream, 3.5 g olive oil, 3 g sunflower oil, 12 g casein, 9 g glucose, 4.5 g beet sugar, 10 g chocolate powder, 150 g water.